Binding Partners for the Thyrotropin Receptor and uses thereof

The present invention is concerned with binding partners (such as monoclonal or recombinant antibodies) for the thyrotropin receptor (TSH receptor or TSHR) and uses thereof.

Thyrotropin or thyroid stimulating hormone (TSH) is a pituitary hormone which plays a key role in regulating the function of the thyroid. Its release is stimulated by the hormone TRH formed in the hypothalamus and TSH controls the formation and release of the important thyroid hormones thyroxine (T4) and tri-iodothyronine (T3). On the basis of a feedback mechanism, the thyroid hormone content of serum controls the release of TSH. The formation of T3 and T4 by thyroid cells is stimulated by TSH by a procedure in which the TSH released by the pituitary binds to the TSH receptor of the thyroid cell membrane.

In Graves' disease (a common autoimmune disorder) TSH receptor antibodies (TRAb) are formed and these autoantibodies bind to the TSH receptor in such a way as to mimic the actions of TSH, stimulating the thyroid gland to produce high levels of thyroid hormones. These autoantibodies are described as having stimulating activity. In some patients, autoantibodies bind to the TSH receptor but do not stimulate thyroid hormone production and are described as having blocking activity. (J Sanders, Y Oda, S-A Roberts, M Maruyama, J Furmaniak, B Rees Smith; "Understanding the thyrotrophin receptor function-structure relationship" Ballière's Clinical Endocrinology and Metabolism; Ed TF Davies 1997; **11**(3): 451-479; pub Ballière Tindall, London).

Measurements of TSH receptor antibodies are important in the diagnosis and management of Graves' disease and other thyroid disorders. Currently three types of assay are used to measure TSH receptor antibodies:-

(a) competitive binding assays which measure the ability of TSH receptor antibodies to inhibit the binding of TSH to preparations of TSH receptor;

(b) bioassays which measure the ability of TSH receptor antibodies to stimulate cells expressing the TSH receptor in culture; and

(c) immunoprecipitation of TSH receptor preparations with TSH receptor antibodies.

Measurement of TSH receptor antibodies using such assays are described in references:-

J Sanders, Y Oda, S-A Roberts, M Maruyama, J Furmaniak, B Rees Smith; "Understanding the thyrotrophin receptor function-structure relationship" Ballière's Clinical Endocrinology and Metabolism; Ed TF Davies 1997; **11**(3): 451-479; pub Ballière Tindall, London.

J Sanders, Y Oda, S Roberts, A Kiddie, T Richards, J Bolton, V McGrath, S Walters, D Jaskólski, J Furmaniak, B Rees Smith; "The interaction of TSH receptor autoantibodies with ^{125}I-labelled TSH receptor"; Journal of Clinical Endocrinology and Metabolism 1999; **84**(10): 3797-3802.

It has been recognised for many years that human monoclonal antibodies to the TSH receptor derived from patients' lymphocytes would be valuable reagents for understanding the pathogenesis of Graves' disease and for developing new methods of measuring TSH receptor antibodies for example as replacements for TSH in competitive binding assays. Also, as the patient's serum TSH receptor antibodies are usually powerful thyroid stimulators (TSH agonists) stimulating human monoclonal TSH receptor antibodies would be valuable for in vivo applications when tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) required stimulation. Furthermore, as some patient serum TSH receptor antibodies are powerful TSH antagonists (blocking antibodies) human monoclonal TSH receptor antibodies which are TSH antagonists would be valuable for in vivo applications when the activity of tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) required inactivation or to be made unresponsive to TSH, TSH receptor antibodies or other stimulators.

It has also been recognised that one of the major advantages of human monoclonal TSH receptor antibodies over TSH in such in vitro and / or in vivo applications would be the relative ease with which antibodies can be manipulated. For example, manipulation of the TSH receptor binding region of the monoclonal antibodies so as to change their characteristics, such as affinity and biological characteristics including their degree of TSH agonist or antagonist activities. Also, monoclonal antibodies will have a much longer half life than TSH in vivo and this may have considerable advantages in certain in vivo applications. Furthermore, the half life of antibodies can be manipulated easily, for example antibody Fab fragments have a much shorter half life than intact IgG. These general properties of TSH receptor antibodies are described in the publications such as B Rees Smith, SM McLachlan, J Furmaniak; "Autoantibodies to the thyrotropin receptor"; Endocrine Reviews 1988; **9**: 106-121; B Rees Smith, KJ Dorrington, DS Munro; "The thyroid stimulating properties of long-acting thyroid stimulator γG-globulin subunits"; Biochimica et Biophysica Acta 1969; **192**: 277-285; KJ Dorrington, DS Munro; "The long acting thyroid stimulator"; Clinical Pharmacology and Therapeutics 1966; **7**: 788-806.

A still further advantage of human monoclonal TSH receptor antibodies could be in their use to identify and provide new types of TSH receptor antibody binding sites. For example by the generation of antibodies to the regions of the human monoclonal TSH receptor antibodies which bind the TSH receptor. Some of the anti-idiotypic antibodies produced in this way could have potential as new ligands for assays of TSH receptor antibodies, TSH and related compounds. Also they may be effective agents in vivo for regulating the action of TSH receptor antibodies, TSH and related compounds.

Other methods of identifying and providing new types of antibody binding sites using monoclonal antibodies are well known. For example by antibody screening of phage-displayed random peptide libraries as described by JC Scott and GP Smith; "Searching for peptide ligands with an epitope library"; Science 1990; **249**(4967): 386-390 and MA Myers, JM Davies, JC Tong, J Whisstock, M Scealy, IR MacKay, MJ Rowley; "Conformational epitopes on the diabetes autoantigen GAD_{65} identified by peptide phage display and molecular modelling"; Journal of Immunology 2000;

165: 3830-3838. Antibody screening of non-peptide compounds and libraries of non-peptide compounds can also be carried out.

New types of TSH receptor antibody binding sites identified and provided using these procedures may also be useful as new ligands in assays for TSH receptor antibodies, TSH and related compounds. Furthermore they may be effective agents in vivo for regulating the action of TSH receptor antibodies, TSH and related compounds.

In view of the potential value of human monoclonal TSH receptor antibodies there have been considerable efforts over many years to produce such antibodies (see for example B Rees Smith, SM McLachlan, J Furmaniak; "Autoantibodies to the thyrotropin receptor"; Endocrine Reviews 1988; **9**: 106-121. However, to date these efforts have been unsuccessful (see for example SM McLachlan, B Rapoport; "Monoclonal, human autoantibodies to the TSH receptor – The Holy Grail and why are we looking for it"; Journal of Clinical Endocrinology and Metabolism 1996; **81**: 3152-3154 and JHW van der Heijden, TWA de Bruin, KAFM Gludemans, J de Kruif, JP Banga, T Logtenberg; "Limitations of the semisynthetic library approach for obtaining human monoclonal autoantibodies to the thyrotropin receptor of Graves' disease"; Clinical and Experimental Immunology 1999; **118**: 205-212).

It is an object of the present invention to provide a binding partner for the TSH receptor capable of interacting with the TSH receptor in a manner comparable to the interaction of TSH receptor autoantibodies with the TSH receptor, in particular it is an object of the present invention to provide human monoclonal antibodies to the TSH receptor exhibiting a comparable interaction therewith as seen with TSH receptor antibodies present in the sera of patients with hyperthyroid Graves' disease and also to provide recombinant preparations thereof. The considerable difficulties of producing human monoclonal TSH receptor antibodies have been overcome in the invention described herein. In particular the successful production of a human monoclonal TSH receptor antibody with the characteristics of the autoantibodies found in the sera of patients with hyperthyroid Graves' disease is described. The human TSH receptor monoclonal antibody we have produced (described herein as hMAb TSHR 1) binds to the TSH receptor with high affinity and in such a way that small amounts of the antibody inhibit labelled TSH binding to the TSH receptor and small amounts act as

powerful thyroid stimulators. Fab fragments of the antibody and recombinant Fab preparations are similarly effective thyroid stimulators and inhibitors of labelled TSH binding as intact IgG. Monoclonal Fab and / or intact IgG can be labelled with ^{125}I or biotin and shown to bind to the TSH receptor. Such binding is inhibited by TSH receptor autoantibodies in patient sera.

There is provided by the present invention, therefore, a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human monoclonal or recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor.

In particular, there is provided by the present invention a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human monoclonal antibody, or one or more fragments thereof, reactive with the TSH receptor.

In particular, there is provided by the present invention a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor.

In particular, there is provided by the present invention a human monoclonal antibody, or one or more fragments thereof, reactive with the TSH receptor.

In particular, there is provided by the present invention a human recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor. Particularly, the present invention provides one or more fragments of a human recombinant antibody reactive with the TSH receptor.

A binding partner according to the present invention, and in particular, a human monoclonal or recombinant antibody reactive with the TSH receptor according to the present invention can be further characterised by its ability to inhibit TSH binding to the TSH receptor, and / or its ability to stimulate the TSH receptor, both of which have been seen to be comparable to the respective inhibitory and stimulatory properties of TSH receptor autoantibodies present in sera obtained from patients with Graves' disease.

More particularly, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a monoclonal or recombinant antibody.

More particularly, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be further characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a monoclonal or recombinant antibody.

In a preferred embodiment of the present invention, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC

90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg;

or one or more fragments of such a monoclonal or recombinant antibody.

In the case where a binding partner according to the present invention comprises or is derived from one or more fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, in particular for example one or more Fab fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, it may be preferred that such a binding partner can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg.

It may also be preferred in the case where a binding partner according to the present invention comprises or is derived from one or more fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, in particular for example one or more Fab fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, that such a binding partner can be characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 50 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 100 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 200 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 400 units of International Standard NIBSC 90/672 per mg.

It may be still further preferred in the case where a binding partner according to the present invention comprises or is derived from one or more fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, in particular for

example one or more Fab fragments of a monoclonal or recombinant antibody reactive with the TSH receptor, that such a binding partner can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 50 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 100 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 200 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 400 units of International Standard NIBSC 90/672 per mg.

In a preferred case the present invention provides a binding partner for the TSH receptor (typically a human monoclonal antibody), which binding partner is capable of binding to the TSH receptor preferably so as to stimulate the TSH receptor and which comprises an antibody V_H domain selected from the group consisting of a V_H domain as shown in SEQ ID NO. 1 and a V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4.

In a first embodiment of the present invention, there is, therefore, provided a binding partner for the TSH receptor (typically a human monoclonal antibody), which binding partner is capable of binding to the TSH receptor preferably so as to stimulate the TSH receptor and which comprises an antibody V_H domain as shown in SEQ ID NO. 1.

In a second embodiment of the present invention there is, therefore, provided a binding partner for the TSH receptor (typically a human monoclonal antibody), which binding partner is capable of binding to the TSH receptor preferably so as to stimulate

the TSH receptor and which comprises an antibody V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4.

It will be appreciated that a binding partner according to the present invention can comprise an antibody V_H domain substantially as hereinbefore described in the absence of an antibody V_L domain. It is known that single immunoglobulin domains, especially V_H domains, are capable of binding target antigens in a specific manner. Alternatively, a binding partner according to the present invention can comprise an antibody V_H domain paired with an antibody V_L domain to provide an antibody binding site comprising both V_H and V_L domains for a TSH receptor employing techniques well known in the art (Biochim. Biophys. Acta, 192 (1969) 277-285; Proc. Natl. Acad. Sci. USA, Vol. 89, pp 10026-10030, November 1992).

In a preferred case the present invention provides, however, a binding partner for the TSH receptor, which binding partner is capable of binding to the TSH receptor preferably so as to stimulate the TSH receptor and which comprises:

an antibody V_H domain selected from the group consisting of:

> a V_H domain as shown in SEQ ID NO. 1 and a V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4; and / or

an antibody V_L domain selected from the group consisting of:

> a V_L domain as shown in SEQ ID NO. 6 and a V_L domain comprising one or more V_L CDRs with an amino acid sequence selected from SEQ ID NO. 7, SEQ ID NO. 8 and SEQ ID NO. 9.

It may be preferred according to the present invention that a binding partner substantially as hereinbefore described comprises an antibody V_H domain substantially as hereinbefore described paired with an antibody V_L domain substantially as hereinbefore described to provide an antibody binding site comprising

both V_H and V_L domains for the TSH receptor, although as discussed further an antibody V_H domain, or an antibody V_L domain, may be independently used to bind a TSH receptor. It will be appreciated, therefore, that a binding partner substantially as hereinbefore described can comprise an antibody V_H domain substantially as hereinbefore described in the absence of an antibody V_L domain. It will also be appreciated, therefore, that a binding partner substantially as hereinbefore described can comprise an antibody V_L domain substantially as hereinbefore described in the absence of an antibody V_H domain. Alternatively, a binding partner substantially as hereinbefore described can comprise an antibody V_H domain paired with an antibody V_L domain substantially as hereinbefore described to provide an antibody binding site comprising both V_H and V_L domains for the TSH receptor.

Preferred embodiments according to the present invention can thus include a binding partner substantially as hereinbefore described comprising an antibody V_H domain as shown in SEQ ID NO. 1 paired with an antibody V_L domain as shown in SEQ ID NO. 6 to provide an antibody binding site, comprising both these V_H and V_L domains for the TSH receptor.

It is further envisaged according to the present invention that V_H domains substantially as hereinbefore described may be paired with V_L domains other than those specifically described herein. It is also further envisaged according to the present invention that V_L domains substantially as hereinbefore described may be paired with V_H domains other than those specifically described herein.

According to a further embodiment of the present invention there is provided a binding partner substantially as hereinbefore described for the TSH receptor, which binding partner is capable of binding to the TSH receptor so as to stimulate the TSH receptor and which can comprise:

an antibody V_H domain comprising:

a V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4; and / or

an antibody V_L domain comprising:

a V_L domain comprising one or more V_L CDRs with an amino acid sequence selected from SEQ ID NO. 7, SEQ ID NO. 8 and SEQ ID NO. 9.

One or more CDRs as referred to above may be taken from the hereinbefore described V_H and V_L domains and incorporated into a suitable framework. For example, the amino acid sequence of one or more CDRs substantially as hereinbefore described may be incorporated into framework regions of antibodies differing from hMAb TSHR 1 specifically disclosed herein, such antibodies thereby incorporating the one or more CDRs and being capable of binding to the TSH receptor, preferably to stimulate the TSH receptor substantially as hereinbefore described. Alternatively, the present invention may provide a polypeptide capable of binding to the TSH receptor so as to stimulate the TSH receptor substantially as hereinbefore described and comprising the primary structural conformation of amino acids as represented by one or more CDRs as specifically described herein, optionally together with further amino acids, which further amino acids may enhance the binding affinity of one or more CDRs as described herein for the TSH receptor or may have substantially no role in affecting the binding properties of the polypeptide for the TSH receptor.

The present invention, also encompasses variants, analogs, derivatives and fragments of the specific human monoclonal antibody described herein, V_H domains, CDRs and polypeptides disclosed herein, which variants, analogs, derivatives and fragments retain the ability to interact with the TSH receptor (such as for example to stimulate the TSH receptor) substantially as hereinbefore described.

The terms "variants", "analogs", "derivatives" and "fragments" as used herein can be characterised as antibodies, antibody fragments or polypeptides which retain essentially the same biological function or activity as a human monoclonal antibody having a V_H domain as shown in SEQ ID NO.1 and a V_L domain as shown in SEQ ID NO.6 and in particular in respect of the binding properties thereof for the TSH receptor. Suitably, variants, analogs, derivatives and fragments, and variants, analogs

and derivatives of the fragments as described herein, have a primary structural conformation of amino acids in which several or a few (such as 5 to 10, 1 to 5 or 1 to 3) amino acid residues of a human monoclonal antibody having a V_H domain as shown in SEQ ID NO.1 and a V_L domain as shown in SEQ ID NO.6 are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions which do not alter or substantially alter the biological activity or function of a human monoclonal antibody having a V_H domain as shown in SEQ ID NO.1 and a V_L domain as shown in SEQ ID NO.6. Conservative substitutions can be preferred as hereinafter described in greater detail.

More particularly, variants, analogs or derivatives of a human monoclonal antibody having a V_H domain as shown in SEQ ID NO.1 and a V_L domain as shown in SEQ ID NO.6 according to the present invention may be ones in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), or ones in which one or more of the amino acid resides includes a substituent group or the like. Such variants, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Most typically, variants, analogs or derivatives are those that vary from a reference human monoclonal antibody having a V_H domain as shown in SEQ ID NO.1 and a V_L domain as shown in SEQ ID NO.6 by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids A, V, L and I; among the hydroxyl residues S and T; among the acidic residues D and E; among the amide residues N and Q; among the basic residues K and R; and among the aromatic residues F and Y.

It will be appreciated that the term fragment as used herein in particular relates to fragments of antibodies specifically as herein described and form an important aspect of the present invention. In this way, a human monoclonal or recombinant antibody as provided by the present invention may be provided as any of the following fragments: (i) the Fab fragment consisting of V_L, V_H, C_L and C_H1 domains; (ii) the Fd fragment consisting of the V_H and C_H1 domains; (iii) the Fv fragment consisting of

the V_L and V_H domains; (iv) the dAb fragment which consists of a V_H domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments; and (vii) single chain Fv molecules (scFv), wherein a V_H domain and a V_L domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site.

Alternatively, a human monoclonal or recombinant antibody according to the present invention may comprise a whole IgG antibody, whereby the antibody includes variable and constant regions.

The present invention also provides a further binding partner capable of binding to the TSH receptor, which can compete for binding to the TSH receptor with a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described, which further binding partner does not comprise TSH. Preferably, this further binding partner may comprise a further antibody having a binding site for an epitope region of the TSH receptor, and which can compete for binding to the TSH receptor with a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described. A suitable such further binding partner can comprise a mouse monoclonal antibody, which can preferably be produced according to techniques substantially as described in the Examples, employing immunisation of mice with TSH receptor by techniques known in the art.

The present invention may also provide a further binding partner capable of binding to the TSH receptor, which can comprise, or is derived from, a human monoclonal or recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor. In particular this further binding partner may comprise a further antibody having a binding site for an epitope region of the TSH receptor, which further antibody is capable of binding to the TSH receptor, and can compete for binding to the TSH receptor with a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described. Suitably such a further binding partner can be derived from a specific binding partner as described herein, hMAb TSHR 1, by suitable mutagenesis techniques, such as spot mutations or the like, so as to obtain a further binding partner for the TSH receptor that can compete

with a binding partner substantially as herein described (such as hMAb TSHR 1) for interaction with the TSH receptor.

Preferably a further binding partner for the TSH receptor can comprise a monoclonal or recombinant antibody and can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments of the antibody. It may also be preferred that such a further binding partner according to the present invention, can be characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg, or one or more fragments of the antibody.

It may also be even more preferred that such a further binding partner of the present invention, can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of

International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg;

or one or more fragments thereof.

A preferred mouse monoclonal antibody providing a further binding partner according to the present invention comprises 9D33 prepared further to the Examples and having amino acid and polynucleotide sequences as illustrated by Figures 9 to 12 and Sequence Listings 19 to 38. According to the present invention, there is, therefore, provided a further binding partner for the TSH receptor (typically a mouse monoclonal antibody), which comprises an antibody V_H domain as shown in SEQ ID NO. 19.

A further binding partner as provided by the present invention can also be characterised as comprising an antibody V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 20, SEQ ID NO. 21 and SEQ ID NO. 22.

It will be appreciated that a further binding partner according to the present invention can comprise an antibody V_H domain substantially as hereinbefore described in the absence of an antibody V_L domain. It is known that single immunoglobulin domains, especially V_H domains, are capable of binding target antigens in a specific manner. Alternatively, a further binding partner according to the present invention can comprise an antibody V_H domain paired with an antibody V_L domain to provide an antibody binding site comprising both V_H and V_L domains for a TSH receptor employing techniques well known in the art (Biochim. Biophys. Acta, 192 (1969) 277-285; Proc. Natl. Acad. Sci. USA, Vol. 89, pp 10026-10030, November 1992).

In a preferred case the present invention provides, however, a further binding partner for the TSH receptor, which further binding partner comprises:

an antibody V_H domain selected from the group consisting of:

a V_H domain as shown in SEQ ID NO. 19 and a V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 20, SEQ ID NO. 21 and SEQ ID NO. 22; and / or

an antibody V_L domain selected from the group consisting of:

a V_L domain as shown in SEQ ID NO. 24 and a V_L domain comprising one or more V_L CDRs with an amino acid sequence selected from SEQ ID NO. 25, SEQ ID NO. 26 and SEQ ID NO. 27.

It may be preferred according to the present invention that a further binding partner substantially as hereinbefore described comprises an antibody V_H domain substantially as hereinbefore described paired with an antibody V_L domain substantially as hereinbefore described to provide an antibody binding site comprising both V_H and V_L domains for the TSH receptor, although as discussed further an antibody V_H domain, or an antibody V_L domain, may be independently used to bind a TSH receptor. It will be appreciated, therefore, that a further binding partner substantially as hereinbefore described can comprise an antibody V_H domain substantially as hereinbefore described in the absence of an antibody V_L domain. It will also be appreciated, therefore, that a further binding partner substantially as hereinbefore described can comprise an antibody V_L domain substantially as hereinbefore described in the absence of an antibody V_H domain. Alternatively, a further binding partner substantially as hereinbefore described can comprise an antibody V_H domain paired with an antibody V_L domain substantially as hereinbefore described to provide an antibody binding site comprising both V_H and V_L domains for the TSH receptor.

Preferred embodiments according to the present invention can thus include a further binding partner substantially as hereinbefore described comprising an antibody V_H domain as shown in SEQ ID NO. 19 paired with an antibody V_L domain as shown in SEQ ID NO. 24 to provide an antibody binding site, comprising both these V_H and V_L domains for the TSH receptor.

It is further envisaged according to the present invention that V_H domains substantially as hereinbefore described may be paired with V_L domains other than those specifically described herein. It is also further envisaged according to the present invention that V_L domains substantially as hereinbefore described may be paired with V_H domains other than those specifically described herein.

According to a further embodiment of the present invention there is provided a further binding partner substantially as hereinbefore described for the TSH receptor, which further binding partner is capable of binding to the TSH receptor so as to inhibit stimulation of the TSH receptor and which can comprise:

an antibody V_H domain comprising:

> a V_H domain comprising one or more V_H CDRs with an amino acid sequence selected from SEQ ID NO. 20, SEQ ID NO. 21 and SEQ ID NO. 22; and / or

an antibody V_L domain comprising:

> a V_L domain comprising one or more V_L CDRs with an amino acid sequence selected from SEQ ID NO. 25, SEQ ID NO. 26 and SEQ ID NO. 27.

One or more CDRs as referred to above may be taken from the hereinbefore described V_H and V_L domains and incorporated into a suitable framework. For example, the amino acid sequence of one or more CDRs substantially as hereinbefore described may be incorporated into framework regions of antibodies differing from 9D33 specifically disclosed herein, such antibodies thereby incorporating the one or more CDRs and being capable of binding to the TSH receptor. Alternatively, the present invention may provide a polypeptide capable of binding to the TSH receptor comprising the primary structural conformation of amino acids as represented by one or more CDRs as specifically described herein, optionally together with further amino acids, which further amino acids may enhance the binding affinity of one or more

CDRs as described herein for the TSH receptor or may have substantially no role in affecting the binding properties of the polypeptide for the TSH receptor.

It will be appreciated that the term fragment as used herein in particular relates to fragments of antibodies specifically as herein described and form an important aspect of the present invention. In this way, a further binding partner according to the present invention may be provided as any of the following fragments: (i) the Fab fragment consisting of V_L, V_H, C_L and C_H1 domains; (ii) the Fd fragment consisting of the V_H and C_H1 domains; (iii) the Fv fragment consisting of the V_L and V_H domains; (iv) the dAb fragment which consists of a V_H domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments; and (vii) single chain Fv molecules (scFv), wherein a V_H domain and a V_L domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site.

Alternatively, a mouse monoclonal or recombinant antibody according to the present invention, such as 9D33, may comprise a whole IgG antibody, whereby the antibody includes variable and constant regions.

There is also provided by the present invention a polynucleotide comprising:

(i) a nucleotide sequence as shown in SEQ ID NO. 10, SEQ ID NO. 11, SEQ ID NO. 12, SEQ ID NO. 13, SEQ ID NO. 15, SEQ ID NO. 16, SEQ ID NO. 17 or SEQ ID NO. 18, encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, as shown in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8 or SEQ ID NO. 9;

(ii) a nucleotide sequence encoding a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described, or encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, of a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described;

(iii) a nucleotide sequence differing from any sequence of (i) in codon sequence due to the degeneracy of the genetic code;

(iv) a nucleotide sequence comprising an allelic variation of any sequence of (i);

(v) a nucleotide sequence comprising a fragment of any of the sequences of (i), (ii), (iii), or (iv) and in particular a nucleotide sequence comprising a fragment of any of the sequences of (i), (ii), (iii), (iv) or (v) and encoding a Fab fragment, a Fd fragment, a Fv fragment, a dAb fragment, an isolated CDR region, F(ab')2 fragments or a scFv fragment, of a human monoclonal antibody substantially as hereinbefore described;

(vi) a nucleotide sequence differing from the any sequence of (i) due to mutation, deletion or substitution of a nucleotide base and encoding a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described, or encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, of a binding partner for the TSH receptor (typically a human monoclonal antibody) substantially as hereinbefore described.

There is also provided by the present invention a polynucleotide comprising:

(i) a nucleotide sequence as shown in SEQ ID NO. 29, SEQ ID NO. 30, SEQ ID NO. 31, SEQ ID NO. 32, SEQ ID NO. 34, SEQ ID NO. 35, SEQ ID NO. 36 or SEQ ID NO. 37, encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, as shown in SEQ ID NO. 19, SEQ ID NO. 20, SEQ ID NO. 21, SEQ ID NO. 22, SEQ ID NO. 24, SEQ ID NO. 25, SEQ ID NO. 26 or SEQ ID NO. 27;

(ii) a nucleotide sequence encoding a further binding partner for the TSH receptor (typically a mouse monoclonal antibody) substantially as hereinbefore described, or encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, of a further binding partner for the TSH

receptor (typically a mouse monoclonal antibody) substantially as hereinbefore described;

(iii) a nucleotide sequence differing from any sequence of (i) in codon sequence due to the degeneracy of the genetic code;

(iv) a nucleotide sequence comprising an allelic variation of any sequence of (i);

(v) a nucleotide sequence comprising a fragment of any of the sequences of (i), (ii), (iii), or (iv) and in particular a nucleotide sequence comprising a fragment of any of the sequences of (i), (ii), (iii), (iv) or (v) and encoding a Fab fragment, a Fd fragment, a Fv fragment, a dAb fragment, an isolated CDR region, F(ab')2 fragments or a scFv fragment, of a mouse monoclonal antibody substantially as hereinbefore described;

(vi) a nucleotide sequence differing from the any sequence of (i) due to mutation, deletion or substitution of a nucleotide base and encoding a further binding partner for the TSH receptor (typically a mouse monoclonal antibody) substantially as hereinbefore described, or encoding an amino acid sequence of an antibody V_H domain, V_L domain, or CDR, of a further binding partner for the TSH receptor (typically a mouse monoclonal antibody) substantially as hereinbefore described.

Variant polynucleotides according to the present invention are suitably at least 70% identical over their entire length to any polynucleotide sequence of (i), most highly preferred are polynucleotides that comprise a region that is at least 80% identical over its entire length to any polynucleotide sequence of (i), polynucleotides at least 90% identical over their entire length to any polynucleotide sequence of (i) are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% identity are especially preferred.

The present invention further provides a biologically functional vector system which carries a polynucleotide substantially as hereinbefore described and which is capable of introducing the polynucleotide into the genome of a host organism.

The present invention also relates to host cells which are transformed with polynucleotides of the invention and the production of binding partners for the TSH receptor of the invention by recombinant techniques. Host cells can be genetically engineered to incorporate polynucleotides and express binding partners for the TSH receptor of the present invention.

The amino acid sequences of hMAb TSHR 1, a human monoclonal antibody according to the present invention, and nucleotide sequences coding therefor, the amino acid sequences of 9D33, a mouse monoclonal antibody which represents a further binding partner according to the present invention, and nucleotide sequences coding therefor, are shown in the Sequence listings as herein after described and can be assigned as follows.

For hMAb TSHR 1:

Amino Acid Sequences

SEQ 1D NO. 1	V_H
SEQ ID NO. 2	V_H CDRI
SEQ ID NO. 3	V_H CDRII
SEQ ID NO. 4	V_H CDRIII
SEQ ID NO. 5	Heavy chain variable and adjacent constant region
SEQ 1D NO. 6	V_L
SEQ 1D NO. 7	V_L CDRI
SEQ 1D NO. 8	V_L CDRII
SEQ 1D NO. 9	V_L CDRIII

Nucleotide Sequences

SEQ ID NO. 10	V_H
SEQ ID NO. 11	V_H CDRI

SEQ ID NO. 12 V_H CDRII
SEQ ID NO. 13 V_H CDRIII.
SEQ ID NO. 14 Heavy chain variable and adjacent constant region
SEQ ID NO. 15 V_L
SEQ ID NO. 16 V_L CDRI
SEQ ID NO. 17 V_L CDRII
SEQ ID NO. 18 V_L CDRIII

For 9D33:

Amino Acid Sequences

SEQ 1D NO. 19 V_H
SEQ ID NO. 20 V_H CDRI
SEQ ID NO. 21 V_H CDRII
SEQ ID NO. 22 V_H CDRIII
SEQ ID NO. 23 Heavy chain variable and adjacent constant region
SEQ 1D NO. 24 V_L
SEQ 1D NO. 25 V_L CDRI
SEQ 1D NO. 26 V_L CDRII
SEQ 1D NO. 27 V_L CDRIII
SEQ ID NO. 28 Light chain variable and adjacent constant region

Nucleotide Sequences

SEQ 1D NO. 29 V_H
SEQ ID NO. 30 V_H CDRI
SEQ ID NO. 31 V_H CDRII
SEQ ID NO. 32 V_H CDRIII
SEQ ID NO. 33 Heavy chain variable and adjacent constant region
SEQ 1D NO. 34 V_L
SEQ 1D NO. 35 V_L CDRI
SEQ 1D NO. 36 V_L CDRII
SEQ 1D NO. 37 V_L CDRIII
SEQ ID NO. 38 Light chain variable and adjacent constant region

The above sequences for hMab TSHR1 can also be seen by reference to Figures 4, 5, 6 and 7, wherein:

Figure 4 shows the hMab TSHR1 heavy chain nucleotide sequence, along with the adjacent constant region, with –

Figure 4a giving the nucleotide sequence *per se*;

Figure 4b giving the nucleotide sequence annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

Figure 5 shows the hMab TSHR1 heavy chain amino acid sequence, along with the adjacent constant region, with –

Figure 5a giving the amino acid sequence *per se*;

Figure 5b giving the amino acid sequence annotated with the CDRI, CDRII, CDRIII and constant regions;

Figure 6 shows the hMab TSHR1 light chain nucleotide sequence, with –

Figure 6a giving the nucleotide sequence *per se*;

Figure 6b giving the nucleotide sequence annotated with the PCR primer, CDRI, CDRII and CDRIII regions;

Figure 7 shows the hMab TSHR1 light chain amino acid sequence, with –

Figure 7a giving the amino acid sequence *per se*;

Figure 7b giving the amino acid sequence annotated with the CDRI, CDRII and CDRIII regions.

It will be appreciated from the above that for the V_H chain of hMab TSHR1 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 4b correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 11, 12 and 13 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 5b correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 2, 3 and 4 respectively. It will also be appreciated from the above that for the V_L chain of hMab TSHR1 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 6b correspond to the V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 16, 17 and 18 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 7b correspond to the V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 7, 8 and 9 respectively.

Analysis of the crystal structure of hMAb TSHR1 Fab (determined by techniques known in the art) enabled refinement of the HC and LC nucleotide sequences determined using PCR primers which are degenerate. In particular, a HC sequencing artefact for nucleotides 115-120 was identified. Sequencing indicated cacgtg (transcribed to amino acids His Val), whereas the crystal structure more reliably indicated amino acids Gln Leu (corresponding bases being cagctg), with the refined sequences being shown in the accompanying Figures and Sequence listings. Crystal structure analysis also enabled refinement of the HC and LC derived amino acid sequences particularly in the degenerate PCR primer region. In the case of the LC aa 2 was found to be Pro by RT-PCR but was Thr from the crystal structure. In the case of the HC aa 2 was found to be Met by RT-PCR but was Val from the crystal structure. Again, these refined sequences are shown in the accompanying Figures and Sequence listings.

The above sequences for 9D33 can also be seen by reference to Figures 9, 10, 11 and 12, wherein:

Figure 9 shows the 9D33 heavy chain nucleotide sequence, along with the adjacent constant region, with –

Figure 9a giving the nucleotide sequence *per se*;

Figure 9b giving the nucleotide sequence annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

Figure 10 shows the 9D33 heavy chain amino acid sequence, along with the adjacent constant region, with –

Figure 10a giving the amino acid sequence *per se*;

Figure 10b giving the amino acid sequence annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

Figure 11 shows the 9D33 light chain nucleotide sequence, with –

Figure 11a giving the nucleotide sequence *per se*;

Figure 11b giving the nucleotide sequence annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

Figure 12 shows the 9D33 light chain amino acid sequence, with –

Figure 12a giving the amino acid sequence *per se*;

Figure 12b giving the amino acid sequence annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions.

It will be appreciated from the above that for the V_H chain of 9D33 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 9b correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 30, 31 and 32 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 10b correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 20, 21 and 22 respectively. It will also be appreciated from the above that for the V_L chain of 9D33 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 11b correspond to the

V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 35, 36 and 37 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in Figure 12b correspond to the V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 25, 26 and 27 respectively.

The present invention also provides a process of providing a human monoclonal antibody to the TSH receptor substantially as hereinbefore described, which process comprises:

(i) providing a source of lymphocytes from a subject, which subject has TSH receptor antibody activity of greater than about 0.04 units of NIBSC 90/672 per mL of serum with respect to inhibition of TSH binding to the TSH receptor;

(ii) isolating lymphocytes from said lymphocyte source of (i);

(iii) immortalising the isolated lymphocytes; and

(iv) cloning the immortalised lymphocytes so as to produce an immortalised colony secreting a human monoclonal antibody to the TSH receptor substantially as hereinbefore described.

Alternatively, a process of providing a human monoclonal antibody to the TSH receptor substantially as hereinbefore described can be defined as a process which comprises:

(i) providing a source of lymphocytes from a subject, which subject has TSH receptor antibody activity of greater than about 0.1 units of NIBSC 90/672 per mL of serum with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor;

(ii) isolating lymphocytes from said lymphocyte source of (i);

(iii) immortalising the isolated lymphocytes; and

(iv) cloning the immortalised lymphocytes so as to produce an immortalised colony secreting a human monoclonal antibody to the TSH receptor substantially as hereinbefore described.

Preferably a process according to the present invention comprises isolating lymphocytes from peripheral blood, thyroid tissue, spleen tissue, lymph nodes or bone marrow, most typically from peripheral blood. Typically, the source of lymphocytes for use in a method according to the present invention can be further characterised as being obtained from a subject having serum TSH receptor antibody levels of greater than about 0.1 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor, or more typically greater than about 0.2 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor, or more typically greater than about 0.3 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor and preferably being in the range of about 0.3 to 0.5 units of NIBSC 90/672 per mL or greater with respect to inhibition of TSH binding to the TSH receptor. Alternatively, or additionally, the source of lymphocytes for use in a method according to the present invention can typically be further characterised as being obtained from a subject having serum TSH receptor antibody levels of greater than about 0.2 units of NIBSC 90/672 per mL with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor, or more typically greater than about 0.5 units of NIBSC 90/672 per mL with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor and preferably being in the range of about 0.5 to 1.0 units of NIBSC 90/672 per mL or greater with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor. It will be appreciated from the above that the immune response to the TSH receptor of a subject from which lymphocytes are isolated should preferably be in a highly active phase.

Preferably a process according to the present invention comprises infecting the isolated lymphocytes with Epstein Barr virus, and suitably the thus immortalised lymphocytes are fused with a mouse / human cell line. Suitably a process according to the present invention further comprises screening the resulting clones for TSH

receptor antibodies, for example by inhibition of ^{125}I TSH binding to the TSH receptor in an assay system which has a sensitivity of at least about 1unit/L of NIBSC 90/672.

The present invention further provides a process of preparing a human recombinant antibody, or one or more fragments thereof, to the TSH receptor, which process comprises cloning and expression of a human monoclonal antibody to the TSH receptor as provided by the present invention by a process substantially as hereinbefore described, or one or more fragments derived therefrom.

The present invention further provides a human monoclonal or recombinant antibody to the TSH receptor obtained by a process substantially as described above. Preferably such an obtained human monoclonal or recombinant antibody to the TSH receptor according to the present invention, can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a human monoclonal or recombinant antibody.

More particularly, it may be preferred that such a human monoclonal or recombinant antibody according to the present invention, can be further characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a human monoclonal or recombinant antibody.

In a preferred embodiment of the present invention, such a human monoclonal or recombinant antibody according to the present invention, can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg;

or one or more fragments of such a human monoclonal or recombinant antibody.

It may also be preferred that one or more fragments of a thus obtained human monoclonal or recombinant antibody according to the present invention, in particular for example one or more Fab fragments thereof, can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg. It may also be preferred that such one or more fragments can be characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 50 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 100 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 200 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 400 units of International Standard NIBSC 90/672 per mg.

More preferably, such one or more Fab fragments can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 50 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 100 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 200 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 400 units of International Standard NIBSC 90/672 per mg.

A process substantially as described above may further comprise a further process stage whereby the obtained human monoclonal or recombinant antibody is subjected to suitable further processing techniques (such as suitable mutagenesis techniques, such as spot mutations or the like), so as to obtain a further binding partner for the TSH receptor that can compete with a binding partner substantially as herein described (such as hMAb TSHR 1) for interaction with the TSH receptor. Such further processing techniques are well known to one of ordinary skill in the art. The present invention further provides a further binding partner to the TSH receptor obtained by such further processing techniques.

Preferably such a further binding partner for the TSH receptor can comprise a monoclonal or recombinant antibody and can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments thereof. It may also be preferred that such a further binding partner according to the present invention, can be characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30

units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg, or one or more fragments thereof.

It may also be even more preferred that such a further binding partner of the present invention, can be characterised by:

(i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg; and

(ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or even more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg;

or one or more fragments thereof.

A binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention may have diagnostic and therapeutic applications.

Accordingly, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in screening methods for detecting autoantibodies to the TSH receptor in patient sera and also in diagnostic methods. In this way, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present

invention can be employed in place of, or in addition to, competitors hitherto described for use in screening methods for detecting autoantibodies to the TSH receptor and also in diagnostic methods. Similarly, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in place of, or in addition to, competitors hitherto described for use in kits for use in detecting autoantibodies to the TSH receptor.

The present invention also provides, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to a TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

A method according to the present invention for the detection of autoantibodies as described above is particularly advantageous in terms of the level of sensitivity that can be achieved by use thereof. This can be further illustrated by reference to the Examples and Figures, where Figure 3a shows a graphical representation of a comparison between an assay for TSHR autoantibodies based on hMAb TSHR1-biotin and earlier assays. The sensitivity of the assay based on hMAb TSHR1-biotin is clearly superior according to concentration of the international standard NIBSC 90/672 detectable. This was confirmed in a study of sera from 72 patients with Graves' disease shown in Figure 3b.

There is further provided by the present invention, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention; and

(d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

The above sensitivity can also be achieved in an assay method or kit according to the present invention by the use of a human or non-human polyclonal antibody to the TSH receptor, TSH or one or more variants, analogs, derivatives or fragments thereof, or a binding partner for the TSH receptor which has an affinity for the TSH receptor of 10^{10}molar^{-1} or greater, which generally exhibit a sufficient affinity for the TSH receptor so that a method or kit of the defined sensitivity is provided. The preparation of such polyclonal antibodies, TSH or one or more variants, analogs, derivatives or fragments thereof, is well known in the art. For example, superactive analogs of TSH are described in Nature, Biotechnology, Volume 14, October 1995, pages 1257-1263, although this article does not disclose the use of such superactive TSH in a method or kit as is now provided by the present invention.

There is further provided by the present invention, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a human or non-human polyclonal antibody to the TSH receptor and a second molecule of said binding pair comprises a binding region with which said polyclonal antibody interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said polyclonal antibody; and

(d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also provided by the present invention a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises TSH or one or more variants, analogs, derivatives or fragments thereof, and a second molecule of said binding pair comprises a binding region with which said TSH or one or more variants, analogs, derivatives or fragments thereof interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said TSH or one or more variants, analogs, derivatives or fragments thereof; and

(d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an

indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also still further provided a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner for the TSH receptor which has an affinity for the TSH receptor of 10^{10}molar^{-1} or greater and a second molecule of said binding pair comprises a binding region with which said binding partner interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner for the TSH; and

(d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also provided by the present invention use of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention, for detecting autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, wherein the interaction of said binding

partner or further binding partner with the TSH receptor is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable.

There is also provided use of a human or non-human polyclonal antibody to the TSH receptor, for detecting autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, wherein the interaction of said polyclonal antibody with the TSH receptor is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable.

There is also provided use of TSH or one or more variants, analogs, derivatives or fragments thereof, for detecting autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, wherein the interaction of said TSH or one or more variants, analogs, derivatives or fragments thereof with the TSH receptor is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable.

There is still further provided use of a binding partner for the TSH receptor which has an affinity for the TSH receptor of 10^{10}molar^{-1} or greater, for detecting autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, wherein the interaction of said binding partner with the TSH receptor is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable.

It will be appreciated that binding molecules of the one or more binding pairs can be antigen-antibody (for example, [TSH receptor or epitope]–[monoclonal or recombinant TSH receptor antibody]), anti-idiotypic antibody-monoclonal or recombinant TSH receptor antibody or novel TSH receptor antibody binding member-

monoclonal or recombinant TSH receptor antibody. Preferably, the binding molecules of the binding pairs are antigen-antibody, namely, [TSH receptor or one or more epitopes thereof]–[monoclonal or recombinant TSH receptor antibody], where the epitopes may be "free standing" or present in a larger scaffold polypeptide or the like.

Preferably, the present invention provides a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to a TSH receptor, said method comprising:

(a) providing said sample of body fluid from said subject;

(b) contacting said sample with (i) a full length TSH receptor, or one or more epitopes thereof or a polypeptide comprising one or more epitopes of a TSH receptor, and (ii) a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention, under conditions that allow interaction of the TSH receptor with autoantibodies produced in response to the TSH receptor, so as to permit said TSH receptor, or said one or more epitopes thereof or said polypeptide, to interact with either autoantibodies to the TSH receptor present in said sample, or said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(c) monitoring the interaction of said TSH receptor, or said one or more epitopes thereof or said polypeptide, with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

In certain embodiments, a method according to the present invention may also employ one or more competitors that compete in the interaction of a polyclonal antibody, TSH or one or more variants, analogs, derivatives or fragments thereof, or a binding partner or further binding partner for the TSH receptor substantially as described above in the specific embodiments of methods as provided by the present invention and the second

molecule of the binding pair, or the TSH receptor, or the one or more epitopes thereof or the polypeptide. Such competitors may comprise TSH, or one or more monoclonals reactive with the TSH receptor, such as mouse monoclonals reactive with the TSH receptor.

Preferably, a method according to the present invention as referred to above, further comprises providing labelling means for a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and where appropriate one or more competitors as described above, suitable labelling means including enzymic labels, isotopic labels, chemiluminescent labels, fluorescent labels, dyes and the like.

The present invention also provides, a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to a TSH receptor, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts;

(b) means for contacting said sample of body fluid from said subject with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(c) means for monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing

an indication of the presence of said autoantibodies to the TSH receptor in said sample.

The present invention also provides a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(b) means for contacting said sample of body fluid from said subject with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention; and

(c) means for monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also provided a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a human or non-human polyclonal antibody to the TSH receptor and a second molecule of said binding pair comprises a binding region with which said polyclonal antibody interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(b) means for contacting said sample of body fluid from said subject with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said polyclonal antibody; and

(c) means for monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also provided a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises TSH or one or more variants, analogs, derivatives or fragments thereof, and a second molecule of said binding pair comprises a binding region with which said TSH or one or more variants, analogs, derivatives or fragments thereof interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(b) means for contacting said sample of body fluid from said subject with said one or more pairs of binding molecules so as to permit said second molecule

of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) TSH or one or more variants, analogs, derivatives or fragments thereof; and

(c) means for monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

There is also provided a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner for the TSH receptor which has an affinity for the TSH receptor of 10^{10}molar^{-1} or greater and a second molecule of said binding pair comprises a binding region with which said binding partner interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

(b) means for contacting said sample of body fluid from said subject with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner for the TSH receptor; and

(c) means for monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

It will be appreciated that binding molecules of the one or more binding pairs can be antigen-antibody (for example, [TSH receptor or epitope]–[monoclonal or recombinant TSH receptor antibody]), anti-idiotypic antibody-monoclonal or recombinant TSH receptor antibody or novel TSH receptor antibody binding member-monoclonal or recombinant TSH receptor antibody. Preferably, the binding molecules of the binding pairs are antigen-antibody, namely, [TSH receptor or one or more epitopes thereof]–[monoclonal or recombinant TSH receptor antibody], where the epitopes may be "free standing" or present in a larger scaffold polypeptide or the like.

The present invention preferably provides a kit for screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said kit comprising:

(a) a full length TSH receptor, or one or more epitopes thereof or a polypeptide comprising one or more epitopes of the TSH receptor;

(b) a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention;

(c) means for contacting said sample of body fluid from said subject, said TSH receptor, or said one or more epitopes thereof or said polypeptide, and said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody), under conditions that allow interaction of the TSH receptor with autoantibodies produced in response to the TSH receptor, so as to permit said TSH receptor, or said one or more epitopes thereof or said polypeptide, to interact with either autoantibodies to a TSH receptor present in said sample, or said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(d) means for monitoring the interaction of said TSH receptor, or said one or more epitopes thereof or said polypeptide, with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

In certain embodiments, a kit according to the present invention may further comprise one or more competitors that compete in the interaction of a polyclonal antibody, TSH or one or more variants, analogs, derivatives or fragments thereof, or a binding partner or further binding partner for the TSH receptor, as respectively defined above, and the second molecule of the binding pair, or the TSH receptor, or the one or more epitopes thereof or the polypeptide. Such competitors may comprise TSH, or one or more monoclonals reactive with the TSH receptor, such as mouse monoclonals reactive with the TSH receptor.

Suitably, a kit as referred to above further comprises labelling means for a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and where appropriate one or more competitors as described above, suitable labelling means being substantially as hereinbefore described.

In the presence of autoantibodies to the TSH receptor, binding of the TSH receptor to a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) in a method or kit as described above will be decreased.

A binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can also be employed in assay methods and kits substantially as described above for TSH and related ligands.

The present invention also provides, therefore, a method of assaying TSH and related ligands, said method comprising:

(a) providing a sample suspected of containing or containing TSH or related ligands;

(b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts;

(c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) TSH or related ligands present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(d) monitoring the interaction of said second molecule of said binding pair with TSH or related ligands present in said sample, thereby providing an indication of the presence of TSH or related ligands in said sample.

The present invention also provides a kit for assaying TSH or related ligands, said kit comprising:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts;

(b) means for contacting a sample suspected of containing or containing TSH or related ligands with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) TSH or related ligands present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

(c) means for monitoring the interaction of said second molecule of said binding pair with TSH or related ligands present in said sample, thereby providing an indication of the presence of TSH or related ligands in said sample.

The present invention also further provides a method of identifying a further binding partner for the TSH receptor, which further binding partner is capable of binding to the TSH receptor and which competes for binding to the TSH receptor with a binding partner for the TSH receptor substantially as hereinbefore described, which further binding partner does not comprise TSH, which method comprises:

(a) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner for the TSH receptor substantially as hereinbefore described and a second molecule of said binding pair comprises a binding region with which said binding partner interacts;

(b) providing a further binding molecule to be assayed as a potential further binding partner for the TSH receptor which competes for binding to the TSH receptor with said first molecule of said binding pair of (a);

(c) contacting said further binding molecule of (b) with said one or more pairs of binding molecules of (a) so as to permit said second molecule of said binding pair of (a) to interact with either (i) said further binding molecule of (b), or (ii) said first molecule of said binding pair of (a); and

(d) monitoring the interaction of said second molecule of said binding pair of (a) with said further binding molecule of (b), and thereby assessing whether said further binding molecule of (b) competes for binding to the TSH receptor with said first molecule of said binding pair of (a).

The present invention also provides a kit for identifying a further binding partner for the TSH receptor, which further binding partner is capable of binding to the TSH receptor and which competes for binding to the TSH receptor with a binding partner

for the TSH receptor substantially as hereinbefore described, which further binding partner does not comprise TSH, which kit comprises:

(a) one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner for the TSH receptor substantially as hereinbefore described and a second molecule of said binding pair comprises a binding region with which said binding partner interacts;

(b) means for contacting said one or more pairs of binding molecules of (a) with a further binding molecule to be assayed as a potential further binding partner for the TSH receptor which competes for binding to the TSH receptor with said first molecule of said binding pair of (a), so as to permit said second molecule of said binding pair of (a) to interact with either (i) said further binding molecule, or (ii) said first molecule of said binding pair of (a); and

(c) means for monitoring the interaction of said second molecule of said binding pair of (a) with said further binding molecule, and thereby assessing whether said further binding molecule competes for binding to the TSH receptor with said first molecule of said binding pair of (a).

A further application of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention is its use to identify and provide new types of TSH receptor antibody binding sites. There is further provided by the present invention, therefore, a process of identifying one or more epitope regions of the TSH receptor, which process comprises contacting a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described with a full length TSH receptor, or one or more fragments thereof, so as to allow interaction of said binding partner or further binding partner for the TSH receptor with said full length TSH receptor, or said one or more fragments thereof, and identifying the amino acids of said full length TSH receptor, or said one or more fragments thereof, with which said binding partner or further binding partner interacts. Suitably, interaction of the binding partner or further binding partner with selected fragments of the TSH receptor and the full length TSH receptor, is analysed,

so as to identify the amino acids of the TSH receptor with which the binding partner interacts.

Furthermore, the present invention allows for generation of antibodies to the regions of a monoclonal TSH receptor antibody according to the present invention which bind the TSH receptor. Such anti-idiotypic antibodies produced in this way could have potential as new ligands for assays of TSH receptor autoantibodies, TSH and related compounds. Also they may be effective agents in vivo for regulating the action of TSH receptor autoantibodies, TSH and related compounds. The present invention further provides, therefore, one or more anti-idiotypic antibodies generated to binding regions of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described, and the preparation thereof is further described by the Examples.

Other methods of identifying and providing new types of antibody binding sites using monoclonal antibodies are well known. For example by antibody screening of phage-displayed random peptide libraries as described by JC Scott and GP Smith; "Searching for peptide ligands with an epitope library"; Science 1990; **249**(4967): 386-390 and MA Myers, JM Davies, JC Tong, J Whisstock, M Scealy, IR MacKay, MJ Rowley; "Conformational epitopes on the diabetes autoantigen GAD_{65} identified by peptide phage display and molecular modelling"; Journal of Immunology 2000; **165**: 3830-3838. Antibody screening of non-peptide compounds and libraries of non-peptide compounds can also be carried out.

New types of TSH receptor antibody binding sites identified and provided using these procedures may also be useful as new ligands in assays for TSH receptor autoantibodies, TSH and related compounds. Furthermore they may be effective agents in vivo for regulating the action of TSH receptor autoantibodies, TSH and related compounds.

A binding partner for the TSH receptor or further binding partner (typically a human monoclonal or recombinant antibody) according to the present invention substantially as hereinbefore described can also be usefully employed in therapy. There is, therefore, further provided by the present invention methods of treatment comprising

administration of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described, pharmaceutical compositions comprising a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described (together with one or more pharmaceutically acceptable carriers, diluents or excipients therefor), and use of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described in the manufacture of a medicament or composition.

A binding partner for the TSH receptor, in particular a human monoclonal antibody to the TSH receptor derived from patients' lymphocytes according to the present invention, is a valuable reagent for understanding the pathogenesis of Graves' disease and for developing new methods of measuring TSH receptor autoantibodies, for example as replacements for TSH in competitive binding assays substantially as hereinbefore described. Also, a stimulating binding partner according to the present invention has in vivo applications when tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) requires stimulation. The present invention provides, therefore, a medicament or composition for use in stimulating thyroid tissue, and / or tissue containing the TSH receptor. In particular, a stimulating binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in oncology, and in particular for use in the diagnosis, management and treatment of thyroid cancer.

Alternatively, a binding partner or further binding partner for the TSH receptor according to the present invention can be a powerful TSH or autoantibody antagonist (blocking antibody) and such a blocking TSH receptor antibody according to the present invention is valuable for in vivo applications when the activity of tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) requires inactivation or to be made unresponsive to TSH, TSH receptor autoantibodies or other stimulators.

There is also provided in combination, a binding partner or further binding partner for the TSH receptor substantially as hereinbefore described, together with one or more

further agents capable of inactivating or rendering unresponsive, tissue containing a TSH receptor, to TSH, TSH receptor autoantibodies or other stimulators. Typically, the one or more further agents act independently of the TSH receptor.

A particular therapeutic application where TSH receptor autoantibody binding requires inactivation or inhibition is in the treatment of disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor, and the use of a blocking antibody which interacts with the TSH receptor, such as 9D33, so as to inhibit TSH receptor autoantibody binding, thus has important therapeutic utility in the treatment of such disease. Treatment of autoimmune disease which requires inhibition of TSH receptor autoantibody binding, such as the above discussed disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor, may alternatively employ an anti-idiotypic antibody to a binding partner or further binding partner as provided by the present invention, and such anti-idiotypic antibodies form a further aspect of the present invention as described herein and further preparatory details thereof are provided by the Examples.

More specifically, therefore, the present invention provides use in the treatment of disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor, of a further binding partner to the TSH receptor, which further binding partner substantially inhibits binding to the TSH receptor of a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described. The present invention further provides use in the manufacture of a medicament for the treatment of disease of the retro orbital tissues of the eye associated with activation and / or stimulation of the TSH receptor, of a further binding partner to the TSH receptor, which further binding partner substantially inhibits binding to the TSH receptor of a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described. There is also provided a method of treating disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor, which method comprises administration to a patient suffering from or susceptible to such disease a therapeutically effective amount of a further binding partner to the TSH receptor, which further binding partner substantially inhibits binding to the TSH receptor of a binding partner for the TSH receptor (typically a human monoclonal or

recombinant antibody) substantially as hereinbefore described. A further binding partner for use in these embodiments of the present invention preferably comprises a blocking antibody which can substantially inhibit binding of a binding partner as provided by the present invention, and as such TSH receptor autoantibody binding, to the TSH receptor, and a preferred such antibody can comprise 9D33 as described herein.

The present invention also provides use of an anti-idiotypic antibody generated to a binding region of a binding partner or further binding partner according to the present invention, in the treatment of disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor. The present invention further provides use of an anti-idiotypic antibody generated to a binding region of a binding partner or further binding partner according to the present invention, in the manufacture of a medicament for the treatment of disease of the retro orbital tissues of the eye associated with activation and / or stimulation of the TSH receptor. There is also provided a method of treating disease of the retro orbital tissues of the eye associated with autoimmunity to the TSH receptor, which method comprises administration to a patient suffering from or susceptible to such disease a therapeutically effective amount of an anti-idiotypic antibody generated to a binding region of a binding partner or further binding partner according to the present invention.

One of the major advantages of a monoclonal antibody as provided by the present invention over TSH in such in vitro and / or in vivo applications is the relative ease with which such antibodies can be manipulated. For example, manipulation of the TSH receptor binding region of a monoclonal antibody according to the present invention so as to change the characteristics thereof, such as affinity and biological characteristics, including the degree of TSH agonist or antagonist activities. Also monoclonal antibodies according to the present invention have a much longer half life than TSH in vivo and this may have considerable advantages in in vivo applications. Furthermore, the half life of the antibodies can be manipulated, for example antibody Fab fragments have a much shorter half life than intact IgG.

Pharmaceutical compositions according to the present invention include those suitable for oral, parenteral and topical administration, although the most suitable route will

generally depend upon the condition of a patient and the specific disease being treated. The precise amount of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described to be administered to a patient will be the responsibility of an attendant physician, although the dose employed will depend upon a number of factors, including the age and sex of the patient, the specific disease being treated and the route of administration substantially as described above.

There is further provided by the present invention a method of stimulating thyroid tissue, and / or tissue containing a TSH receptor, which method comprises administering to a patient in need of such stimulation a diagnostically or therapeutically effective amount of a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described.

The present invention also provides in combination, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) substantially as hereinbefore described, together with one or more further agents capable of stimulating thyroid tissue, and / or tissue containing a TSH receptor, for simultaneous, separate or sequential use in stimulating thyroid tissue, and / or tissue containing a TSH receptor. Preferably the one or more further agents comprise recombinant human TSH and / or one or more variants, analogs, derivatives or fragments thereof, or variants, analogs or derivatives of such fragments. Alternatively, the one or more further agents can act independently of binding to the TSH receptor.

A binding partner for the TSH receptor or further binding partner (typically a human monoclonal or recombinant antibody) according to the present invention can also be employed as a replacement source for patient serum required to contain TSH receptor antibody or antibodies for use in commercial kits. Furthermore, a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) can be provided according to the present invention in a preparation required to comprise a defined concentration of TSH receptor antibody or antibodies, and in this way there can be provided a preparation with a defined activity, such as stimulatory activity, with respect to the TSH receptor. Optionally, such a

preparation may further comprise one or more further human monoclonal antibodies, such as monoclonal antibodies to GAD, TPO or the like.

The following illustrative explanations are provided to facilitate understanding of certain terms used herein. The explanations are provided as a convenience and are not limitative of the invention

BINDING PARTNER FOR A TSH RECEPTOR, describes a molecule having a binding specificity for the TSH receptor. A binding partner as described herein may be naturally derived or wholly or partially synthetically produced. Such a binding partner has a domain or region which specifically binds to and is therefore complementary to one or more epitope regions of the TSH receptor. In particular, a binding partner as described herein can be a monoclonal or recombinant antibody to the TSH receptor, and more particularly can be a human monoclonal or recombinant antibody to the TSH receptor.

C DOMAIN denotes a region of relatively constant amino acid sequence in antibody molecules.

CDR denotes complementarity determining regions which are present on both heavy and light chains of antibody molecules and represent regions of most sequence variability. CDRs represent approximately 15 to 20% of variable domains and represent antigen binding sites of an antibody.

FR denotes framework regions and represent the remainder of the variable light domains and variable heavy domains not present in CDRs.

HC denotes part of a heavy chain of an antibody molecule comprising the heavy chain variable domain and the first domain of an IgG constant region.

HOST CELL is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

IDENTITY, as known in the art, is the relationship between two or more polypeptide sequences, or two or more polynucleotide sequences, as determined by comparing the sequences.

LC denotes a light chain of an antibody molecule.

NIBSC 90/672 is an International Standard for thyroid stimulating antibody. The International Standard for thyroid stimulating activity consists of a batch of ampoules containing freeze dried plasma proteins from a single human patient with high TSH receptor autoantibodies. The preparation has been evaluated in an international collaborative study and shown to possess both thyroid stimulating and thyroid receptor binding activity. At the 46th meeting in 1995, the Expert Committee on Biological Standardization of WHO established the preparation coded 90/672 as the International Standard for thyroid stimulating antibody. Each ampoule contains freeze-dried residue of 1.0ml of a solution containing 0.02M phosphate buffer, dialysed human plasma proteins and 0.1 International Units (100 milli-International Units) per ampoule by definition.

STIMULATION OF A TSH RECEPTOR by a human monoclonal antibody as described herein denotes the ability thereof to bind to a TSH receptor and to thereby effect, for example, production of cyclic AMP as a result of such binding to the TSH receptor. Such stimulation is analogous to the responses seen on binding of TSH, or TSH receptor autoantibodies, to the TSH receptor and in this way a human monoclonal antibody as described herein essentially provides the same or similar binding responses as seen with TSH, or TSH receptor autoantibody, binding to a TSH receptor.

V DOMAIN denotes a region of highly variable amino acid sequence in antibody molecules.

V_HDOMAIN denotes variable regions or domains in heavy chains of antibody molecules.

V_LDOMAIN denotes variable regions or domains in light chains of antibody molecules.

The present invention will now be illustrated by the following Figures and Examples, which do not limit the scope of the invention in any way.

Examples

MATERIALS & METHODS

Lymphocyte isolation and cloning of human monoclonal TSH receptor autoantibodies

Blood was obtained from a patient with Graves' disease and Type 1 diabetes mellitus who had high levels of serum autoantibodies to the TSH receptor (TRAb). Ethical Committee approval was obtained for the studies. Peripheral blood lymphocytes were isolated on Ficoll–Paque (Amersham Biosciences; Chalfont St Giles, HP8 4SP, UK) from a 20mL blood sample and then infected with Epstein Barr virus (EBV) (European Collection of Cell Cultures – ECACC; Porton Down, SP4 0JG, UK) and cultured on mouse macrophage feeder layers as described before (N Hayakawa, LDKE Premawardhana, M Powell, M Masuda, C Arnold, J Sanders, M Evans, S Chen, JC Jaume, S Baekkeskov, B Rees Smith, J Furmaniak; "Isolation and characterization of human monoclonal autoantibodies to glutamic acid decarboxylase"; Autoimmunity 2002; **35**: 343-355). EBV immortalised B lymphocytes were then fused with the mouse/human hybrid cell line K6H6/B5 (WL Carroll, K Thilemans, J Dilley, R Levy; "Mouse x human heterohybridomas as fusion partners with human B cell tumors"; Journal of Immunological Methods 1986; **89**: 61-72) and cloned two times by limiting dilution at 5 cells/well and a final time at ½ cell/well to obtain a single colony (BJ Bolton, NK Spurr. "B-lymphocytes" In: RI Freshney, MG Freshney (eds). Culture of immortalized cells. Wiley-Liss, New York 1996; 283-297). The original wells and subsequent clones were screened for TSH receptor autoantibody by inhibition of ^{125}I-TSH binding to solubilised TSH receptor (see below). The single clones producing TSH receptor autoantibodies were grown up in tissue culture flasks.

Production, purification and labelling of monoclonal TSH receptor antibody preparations

Mouse TSH receptor MAbs were produced as described before (Y Oda, J Sanders, M Evans, A Kiddie, A Munkley, C James, T Richards, J Wills, J Furmaniak, B Rees Smith; "Epitope analysis of the human thyrotropin (TSH) receptor using monoclonal antibodies"; Thyroid 2000; **10**: 1051-1059) and were also prepared from mice immunised with full length TSHR cDNA cloned in pcDNA3.1 (UA Hasan, AM Abai, DR Harper, BW Wren, WJW Morrow; "Nucleic acid immunization: Concepts and techniques associated with third generation vaccines"; Journal of Immunological Methods 1999; **229**: 1-22).

IgGs were purified from tissue culture supernatants using affinity chromatography on Prosep A (Millipore UK Ltd.; Watford, WD18 8YH, UK) according to the manufacturer's instructions and purity assessed by SDS-polyacrylamide gel electrophoresis (PAGE).

Human heavy chain isotype was determined using a radial diffusion assay (The Binding Site; Birmingham, B29 6AT, UK). Human light chain isotype was determined using Western blotting with anti-human kappa chain and anti human lambda chain specific mouse monoclonal antibodies (Sigma-Aldrich Company Ltd; Gillingham, SP8 4XT, UK).

The purified IgG preparations were treated with mercuripapain (Sigma-Aldrich) at an enzyme/protein ratio of between 1:10 and 1:100 (depending on the particular monoclonal antibody) and passed through a Prosep A column to remove any intact IgG or Fc fragment from the Fab preparation (Y Oda, J Sanders, S Roberts, M Maruyama, R Kato, M Perez, VB Petersen, N Wedlock, J Furmaniak, B Rees Smith; "Binding characteristics of antibodies to the TSH receptor"; Journal of Molecular Endocrinology 1998; **20**: 233-244). Intact IgG was undetectable by SDS-PAGE in the Fab preparations. IgG and Fab preparations of the monoclonal antibodies were labelled with ^{125}I as described previously (Y Oda, J Sanders, S Roberts, M Maruyama, R Kato, M Perez, VB Petersen, N Wedlock, J Furmaniak, B Rees Smith; "Binding characteristics of antibodies to the TSH receptor"; Journal of Molecular

Endocrinology; 1998; **20**: 233-244). IgG preparations were labelled with biotin hydrazide (Pierce Rockford IL61105 USA) according to the manufacturers instructions. Crystals of Fab fragments of the human monoclonal TSH receptor autoantibody were obtained and their crystal structure determined using standard techniques.

Patients

Sera from patients with Graves' disease of different disease duration were studied. The patients' sera studied showed inhibition of ^{125}I-labelled TSH binding to the TSH receptor (see below). In addition, sera from 2 patients with Addison's disease (A1 and A2) and high levels of autoantibodies to 21-OH (113 and 1970 units per mL, RSR kit) and sera from 2 patients with type 1 diabetes mellitus (D1 and D2) with high levels of GAD_{65} (3700 and 37.5 units per mL; RSR kit) were studied. Informed consent for the study was obtained from the patients. Sera from healthy blood donors (purchased from Golden West Biologicals, Vista, CA 92083, USA) were also studied. TRAb first international standard preparation (90/672) was obtained from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, EN6 3QH, UK).

Inhibition of ^{125}I-TSH and ^{125}I-mouse TSHR MAb binding to the TSH receptor

Binding inhibition assays were carried out using TSH receptor coated tubes as described previously (J Sanders, Y Oda, S Roberts, A Kiddie, T Richards, J Bolton, V McGrath, S Walters, D Jaskolski, J Furmaniak, B Rees Smith; "The interaction of TSH receptor autoantibodies with ^{125}I-labeled TSH receptor"; Journal of Clinical Endocrinology and Metabolism 1999; **84**: 3797-3802) (reagents from RSR Ltd). Briefly, 100μL of sample (tissue culture supernatant, purified IgG or Fab fragment, patient serum or NIBSC 90/672 standards) were incubated in TSH receptor coated tubes at room temperature for 2 hours with gentle shaking. After aspiration, the tubes were washed twice with 1 mL of assay buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.8, 0.1% Triton X-100) before addition of 100μL of ^{125}I-TSH or ^{125}I-MAb ($5x10^4$ cpm) and incubation at room temperature for 1 hour with shaking. The tubes were then washed twice with 1mL of assay buffer, aspirated and counted in a gamma counter.

Inhibition of binding was calculated as:-

$$100 \times 1 - \frac{\text{cpm bound in the presence of test material}}{\text{cpm bound in the presence of control material}}$$

Control materials used were culture medium, a pool of healthy blood donor sera or as otherwise indicated.

Analysis of thyroid stimulating activities

The ability of monoclonal autoantibody preparations and patient sera to stimulate the production of cyclic AMP (or cAMP) in CHO cells expressing hTSH receptor (approximately 50,000 receptors per cell) (Y Oda, J Sanders, S Roberts, M Maruyama, R Kato, M Perez, VB Petersen, N Wedlock, J Furmaniak, B Rees Smith; "Binding characteristics of antibodies to the TSH receptor"; Journal of Molecular Endocrinology 1998; **20**: 233-244) were carried out according to the method of R Latif, P Graves, TF Davies; "Oligomerization of the human thyrotropin receptor"; Journal of Biological Chemistry 2001; **276**: 45217-45224. Briefly, CHO cells were seeded into 96 well plates (30,000 cells per well) and incubated for 24 hours in DMEM (Invitrogen Ltd; Paisley PA4 9RF, UK) containing 10% fetal calf serum. Culture was then continued in DMEM without fetal calf serum for a further 24 hours. The DMEM was then removed and test TSH, IgG, Fab and serum (100µl diluted in NaCl free Hank's Buffered Salts solution containing 1g/L glucose, 20 mmol/L Hepes, 222 mmol/L sucrose, 15 g/L bovine serum albumin (BSA) and 0.5 mmol/L 3 isobutyl-1-methyl xanthine pH 7.4) added and incubated for 1 hour at 37°C. After removal of the test solutions, cells were lysed and assayed for cyclic AMP using a Biotrak enzyme immunoassay system from Amersham Biosciences; Chalfont St Giles, HP8 4SP, UK. In some experiments, the ability of patient sera and mouse monoclonal antibodies to the TSHR to inhibit the stimulating activity of TSH or hMAb TSHR1 was assessed. This was carried out by comparing (a) the stimulatory effects of TSH or hMAb TSHR1 alone with (b) the stimulatory effects of TSH or hMAb TSHR1 in the presence of patient sera or mouse monoclonal antibody.

Variable Region Gene Analysis

Total RNA was prepared from 1 x 10^7 cells of a TSH receptor autoantibody producing clone using the acid phenol guanidine method (P Chomczynski, N Sacchi; "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction"; Analytical Biochemistry 1987; **162**: 156-159) and mRNA prepared using oligo dT magnetic beads (Dynal Biotech Ltd; Wirral, CH62 3QL, UK). RT-PCR reactions were performed using reagents from Invitrogen Ltd; Paisley PA4 9RF, UK.

Sense strand oligonucleotide primers were designed using the sequences recommended by the Medical Research Council's V-base database (www.mrc-cpe.cam.ac.uk). Antisense primers specific for human IgG1 heavy chain and lambda light chain were based on constant region encoding DNA sequences. Both sense and antisense primers included additional 5' restriction endonuclease site sequences to facilitate cloning of PCR products. IgG1 heavy chain and lambda light chain RT-PCR reactions were performed using the complete panel of appropriate primers. All primers were synthesized by Invitrogen Ltd. The RT reaction took place at 50°C for 10 minutes followed immediately by 40 cycles of PCR (15 sec 94°C, 30 sec 55°C, 30 sec 72°C). RT-PCR products were cloned into pUC18 and DNA prepared using the Wizard kit from Promega UK Ltd; Southampton SO16 7NS, UK and sequenced by the Sanger-Coulson method (F Sanger, S Nicklen, AR Coulson; "DNA sequencing with chain terminating inhibitors"; Proceedings of the National Academy of Sciences of the USA 1977; 74: 5463-5467). V region sequences were compared with available sequences of human Ig genes using Ig blast (www.ncbi.nlm.nih.gov/igblast/igblast.cgi).

Immunoprecipitation Assay (IPA)

The cDNA encoding full length TSH receptor was placed downstream of the T7 promoter in pYES2 (Invitrogen) and used in an in vitro TnT system (Promega UK Ltd) to produce TSH receptor labelled with ^{35}S-methionine as previously described (L Prentice, J Sanders, M Perez, R Kato, J Sawicka, Y Oda, D Jaskolski, J Furmaniak, B Rees Smith; "Thyrotropin (TSH) receptor autoantibodies do not appear to bind to the TSH receptor produced in an in vitro transcription/translation system"; Journal of

Clinical Endocrinology and Metabolism 1997; **82**: 1288-1292). Briefly 50µL ^{35}S-labelled TSH receptor (25 000 – 30 000 cpm) diluted in HSB (150 mmol/L Tris-HCL pH 8.3, 200 mmol/L NaCl and 10 mg/mL bovine serum albumin containing 1% Tween 20) were added to duplicate 50µL aliquots of diluted test sample and incubated for 2 hours at room temperature. Immune complexes were then precipitated by addition of protein A sepharose (Sigma-Aldrich) and counted in a scintillation counter.

TSH receptor preparations and western blotting

Full-length human TSH receptor was expressed in CHO-K1 cells, extracted with 1% Triton X-100 and purified by TSH receptor monoclonal antibody affinity chromatography as described previously (Y Oda, J Sanders, M Evans, A Kiddie, A Munkley, C James, T Richards, J Wills, J Furmaniak, B Rees Smith; "Epitope analysis of the human thyrotropin (TSH) receptor using monoclonal antibodies"; Thyroid 2000; **10**: 1051-1059).

The purified CHO cell produced TSH receptor was run on 9% SDS-PAGE gels, blotted onto nitrocellulose and reacted with test antibodies as described previously (Y Oda, J Sanders, M Evans, A Kiddie, A Munkley, C James, T Richards, J Wills, J Furmaniak, B Rees Smith; "Epitope analysis of the human thyrotropin (TSH) receptor using monoclonal antibodies"; Thyroid 2000; **10**: 1051-1059).

Epitope analysis using TSH receptor peptides

Twenty six peptides each 25aa long covering the whole of the extracellular domain of the human TSH receptor were kindly provided by Dr J Morris (JC Morris, ER Bergert, DJ McCormick; "Structure-function studies of the human thyrotropin receptor. Inhibition of binding of labeled thyrotropin (TSH) by synthetic human TSH receptor peptides"; Journal of Biological Chemistry 1993; **268**: 10900-10905). A human 21-OH peptide (C1, SSSRVPYKDRARLPL) which binds to an M21-OH5 MAb (S Chen, J Sawicka, L Prentice, JF Sanders, H Tanaka, V Petersen, C Betterle, M Volpato, S Roberts, M Powell, B Rees Smith, J Furmaniak; "Analysis of

autoantibody epitopes on steroid 21-hydroxylase using a panel of monoclonal antibodies"; Journal of Clinical Endocrinology and Metabolism 1998; **83**: 2977-2986) was used as a positive control and a human monoclonal antibody to GAD_{65} (N Hayakawa, LDKE Premawardhana, M Powell, M Masuda, C Arnold, J Sanders, M Evans, S Chen, JC Jaume, S Baekkeskov, B Rees Smith, J Furmaniak; "Isolation and characterization of human monoclonal autoantibodies to glutamic acid decarboxylase"; Autoimmunity 2002; **35**: 343-355) was used as a negative control. The peptide ELISA was carried out as described previously (Y Oda, J Sanders, M Evans, A Kiddie, A Munkley, C James, T Richards, J Wills, J Furmaniak, B Rees Smith; "Epitope analysis of the human thyrotropin (TSH) receptor using monoclonal antibodies"; Thyroid 2000; **10**: 1051-1059).

Interaction of monoclonal TSHR autoantibody preparations with the TSH receptor coated onto plastic tubes or ELISA plate wells

(a) ^{125}I-labelled autoantibody

Test samples including patient sera (100µL) were incubated in TSH receptor coated tubes (RSR Ltd.) at room temperature for 2 hours with gentle shaking. After aspiration, the tubes were washed twice with 1mL of assay buffer before addition of 100µL of labelled autoantibody preparation (30,000 cpm) and incubation at room temperature for 1 hour with shaking. The tubes were then washed twice with 1mL of assay buffer, aspirated and counted in a gamma counter. Inhibition of ^{125}I-labelled autoantibody binding was calculated using the formula as for inhibition of TSH binding (see above).

(b) Biotin labelled monoclonal autoantibody and biotin labelled TSH

The procedure described previously (J Bolton, J Sanders, Y Oda, C Chapman, R Konno, J Furmaniak and B Rees Smith; "Measurement of thyroid-stimulating hormone receptor autoantibodies by ELISA; Clinical Chemistry, 1999; **45**: 2285-2287) was used. Briefly, test samples including patient sera (75µL) were incubated in TSH receptor coated ELISA plate wells (RSR Ltd) for 2 hours with shaking (200

shakes per minute) on an ELISA plate shaker. Test samples were then removed and the wells washed once with assay buffer. Biotin-labelled monoclonal TSH receptor autoantibody (1 ng in 100μL) or biotin labelled porcine TSH (RSR Ltd; 5 ng in 100μL) were then added and incubation continued for 25 minutes at room temperature without shaking. The wells were washed once, 100μL of streptavidin-peroxidase (RSR Ltd; 10 ng in 100μL) added and incubation continued for 20 minutes at room temperature without shaking. The wells were then washed 3 times, the peroxidase substrate tetramethyl benzidine (RSR Ltd; 100μL) added. After incubation for 30 minutes at room temperature without shaking 50μL of 0.5M H_2SO_4 was added to stop the substrate reaction and the absorbance of each well read at 450nm on an ELISA plate reader. Inhibition of biotinylated MAb or TSH binding was expressed as an index calculated as:-

$$100 \times 1 - \frac{\text{test sample absorbance at 450nm}}{\text{negative serum control absorbance at 450nm}}$$

Scatchard analysis of monoclonal autoantibody binding to TSH receptor coated tubes

Unlabelled IgG or Fab in 50μL of assay buffer and 50μL of ^{125}I-labelled hMAb IgG or Fab (30,000 cpm in assay buffer) were incubated for 2 hours at room temperature with gentle shaking, washed twice with 1mL of assay buffer and counted in a gamma counter. The concentration of IgG or Fab bound vs bound/free was plotted (G Scatchard; "The attraction of proteins for small molecules and ions"; Annals of the New York Academy of Sciences 1949; **51**: 660-672) to derive the association constants.

Binding of TSH receptor to tubes coated with monoclonal TSH receptor autoantibodies

Test samples including patient sera (100μL) and detergent solubilised TSH receptor (20μL) were incubated for 1 hour at room temperature. Duplicate 50μL aliquots of the incubation mixture were then added to plastic tubes (Nunc Maxisorp) which had been coated with monoclonal TSH receptor autoantibody Fab (200μL of 10μg/mL

overnight at 4°C followed by washing and post coating). After incubation for 1 hour at room temperature with gentle shaking, the tubes were washed, 100µL (40,000 cpm) of ^{125}I-labelled TSH receptor C terminal monoclonal antibody 4E31 (J Sanders, Y Oda, A Kiddie, T Richards, J Bolton, V McGrath, S Walters, D Jaskolski, J Furmaniak, B Rees Smith; "The interaction of TSH receptor autoantibodies with ^{125}I-labelled TSH receptor"; Journal of Clinical Endocrinology and Metabolism 1999; **84**: 3797-3802) added and incubation continued for a further 1 hour with gentle shaking. Then tubes were washed and counted for ^{125}I.

Cloning and expression of recombinant hMAb TSHR1 Fab in E coli

The hMAb TSHR1 heavy chain RT-PCR product (see Variable Region Gene Analysis section) was cut with XhoI and SpeI restriction endonucleases and the hMAb TSHR1 light chain PCR product was cut with SacI and XbaI restriction endonucleases and both heavy and light chain cDNAs cloned into the Immunozap H/L vector (Stratagene Europe; Amsterdam, Netherlands) (I Matthews, G Sims, S Ledwidge, D Stott, D Beeson, N Willcox, A Vincent; "Antibodies to acetylcholine receptor in parous women with myasthenia: evidence for immunization by fetal antigen"; Laboratory Investigation 2002; 82: 1-11) under the control of the lacZ promoter. Plasmid DNA was prepared using the Qiagen midi plasmid purification kit (Qiagen Ltd; Crawley, RH10 9AX, UK) and the presence of hMAb TSHR1 heavy and light chain cDNAs confirmed by sequencing using the Sanger-Coulson method (F Sanger, S Nicklen, AR Coulsen; "DNA sequencing with chain terminating inhibitors"; Proceedings of the National Academy of Sciences of the USA 1977; 74: 5463-5467). Plasmid DNA was transformed into 2 different E coli strains (a) XL1-Blue MRF' (Stratagene) and (b) HB2151 (Amersham Biosciences) and grown overnight at 37°C on LB ampicillin (Tryptone 10g/L, Yeast Extract 5g/L, NaCl 10g/L, 100µg/mL final concentration Ampicillin) agar plates (15g/L agar). Precultures (one colony in 3mL LB ampicillin + 1% glucose) were grown overnight at 37°C with shaking. Production of the recombinant Fab is inhibited in the presence of glucose. Precultures after overnight incubation were diluted 1/100 (0.5mL in 50mL LB ampicillin) and grown at 37°C until the OD_{600} was between 0.4-0.6. These cultures were placed at 30°C with shaking for 20 minutes. Thereafter isopropyl-β-D thiogalactoside (IPTG) was added

to a final concentration of 1mmol/L and cultures continued to be incubated overnight (16 hours) at 30°C with shaking. The cultures were then centrifuged at 3000rpm for 30 minutes at 4°C and the culture supernatants and pellets recovered. The pellets, were resuspended in 1 mL of ice cold TES buffer (0.2 mol/L Tris-HCl pH 8.0, 0.5 mol/L EDTA and 0.5 mol/L sucrose) by vortexing. A further 1.5 mL of ice cold TES buffer diluted 5x in H_2O was added, the mixture vortexed again and incubated on ice for 30 minutes then recentrifuged to give a second supernatant or periplasmic fraction (PF). The culture supernatant and PF were filtered through a 0.45μm filter and dialysed overnight into 10mmol/L Tris pH 7.5, 50mmol/L NaCl. Culture supernatant or PF from untransformed XL1-Blue MRF' and HB2151 cells and XL1-Blue MRF' transformed with hMAb TSHR1 plasmid (XL1-Blue MRF'/hMAb TSHR1) and HB2151 transformed with hMAb TSHR1 plasmid (HB2151/hMAb TSHR1) grown with glucose without IPTG ie non-induced were also prepared. The culture supernatants and PF were assayed for (a) their ability to inhibit TSH binding to the TSHR, (b) their ability to stimulate cyclic AMP production in CHO cells expressing TSHR, and (c) total recombinant Fab concentration by radioimmunoassay. In this assay, calibrators and test materials including culture supernatants and PF (100 μL in duplicate) diluted in assay buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.8, 0.1% Triton X-100) were incubated for 1 hour at room temperature in plastic tubes coated with Fab specific goat anti human IgG (from Sigma Aldrich, Poole, BH12 4QH, UK). The tubes were then washed with assay buffer (2 x 1 mL) and 100 μL of ^{125}I-labelled hMAb TSHR1 Fab (30,000 cpm) added followed by incubation at room temperature. After 1 hour, the tubes were washed again (2 x 1mL) and counted for ^{125}I. The counts bound were plotted against concentration of Fab (hybridoma produced hMAb TSHR1 Fab) in the calibrators (5-500 ng/mL) and the concentration of recombinant Fab in the various test materials read off this calibration curve. The detection limit for this assay was 5-10 ng/mL of Fab.

Cloning and expression of recombinant 4B4 (a human MAb to glutamic acid decarboxylase or GAD) and recombinant hybrid Fabs (mixed HC and LC of hMAb TSHR1 and 4B4)

Recombinant 4B4 Fab (4B4 is described in detail by N Hayakawa, LDKE Premawardhana, M Powell, M Masuda, C Arnold, J Sanders, M Evans, S Chen, JC Jaume, S Baekkeskov, B Rees Smith, J Furmaniak. Isolation and characterization of human monoclonal antibodies to glutamic acid decarboxylase. Autoimmunity 2002 volume 35 pp 343-355) and recombinant hybrid Fabs were produced by cloning the respective HC and LC into Immunozap H/L vector and expressed in HB2151 cells as described for recombinant hMAb TSHR1 Fab. The culture supernatants and periplasmic fraction were assayed for their ability to (a) inhibit TSH binding to the TSHR (b) to stimulate cyclic AMP production in CHO cells expressing the TSHR and (c) for total recombinant Fab concentration as described above. In addition, GAD Ab activity was assessed as described below.

Measurement of recombinant GAD Ab Fab activity in culture supernatants and periplasmic fractions

An assay based on the ability of GAD Ab Fab preparation to inhibit the binding of ^{125}I-labelled GAD (from RSR Ltd, Cardiff, CF23 8HE, UK) to the human monoclonal antibody to GAD (4B4) was used. In the assay, test samples diluted in GAD Ab assay buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 1% v/v Tween 20, 1 g/L bovine serum albumin and 0.5 g/L NaN_3) were incubated (50 µL in duplicate with ^{125}I-labelled GAD (30,000 cpm in 50 µL of GAD Ab assay buffer) for 1 hour at room temperature. Then, 50 µL of 4B4 IgG (0.1 µg/mL in GAD Ab assay buffer) was added and incubation continued for 24 hours at room temperature. Solid phase protein A (50 µL in GAD Ab assay buffer; from RSR Ltd) was then added to precipitate complexes of 4B4 IgG-^{125}I-labelled GAD (protein A does not react with complexes of Fab and ^{125}I-labelled GAD). After allowing the reaction with protein A to proceed for 1 hour at room temperature, the precipitates were pelleted by centrifugation (1500 g for 30 minutes at 4 °C), washed with 1 mL of GAD Ab assay buffer and counted for ^{125}I. ^{125}I-labelled GAD binding in the absence of 4B4 IgG was 4-5% of the total cpm added.

Production of anti-idiotypic antibodies to hMAb TSHR1

6-8 week old BALB/c mice were immunised intraperitoneally with 50 μg hMAb TSHR1 Fab in complete Freunds adjuvant followed by a second injection with 50 μg hMAb TSHR1 Fab in incomplete Freunds adjuvant after 25 days and a further injection of 50 μg hMAb TSHR1 Fab 4 days before removal of the spleen. The spleen cells from antibody positive mice (see below) were fused with a mouse myeloma cell line and monoclonal antibodies isolated as above for TSHR MAbs. The levels of anti-idiotypic antibody in the mouse sera and cell culture wells were measured by inhibition of ^{125}I-hMAb TSHR1 Fab binding to TSHR coated tubes. In particular, duplicate 60 μL aliquots of test sample (diluted in assay buffer: 50 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.8, 0.1% Triton X-100) were incubated with 60 μL of ^{125}I-hMAb TSHR1 Fab (30 000 cpm diluted in assay buffer) for 1 hour at room temperature. 100 μL of the mixture was transferred to duplicate TSHR coated tubes (RSR Ltd) with 20 μL start buffer (see above) and incubation continued for a further two hours at room temperature with shaking. The tubes were then washed with 2 x 1 mL of assay buffer and counted for ^{125}I. The presence of anti-idiotypic antibodies reactive with hMAb TSHR1 was evident from the ability of test samples to inhibit the binding of labelled hMAb TSHR1 Fab to the TSHR coated tubes.

RESULTS

Lymphocytes (30 x 10^6) obtained from 20mL of patient's blood were plated out at 1 x 10^6 per well on a 48 well plate with 200μL of EBV supernatant on feeder layers of mouse macrophages. On day 11 post EBV infection the supernatants were monitored for inhibition of ^{125}I-TSH binding. One well was found to be positive for inhibition of binding, the levels of inhibition increasing to greater than 90% inhibition by day 16 and stayed at that level until day 24, after which time they decreased. The cultures were expanded and fused with K6H6/B5 cells on day 21, 23, 26 and 27 post EBV infection; in total 7 fusion experiments were carried out. Each fusion was plated across 3 x 96 well plates (ie 21 plates in total) and one well, stably producing antibodies with ^{125}I-TSH binding inhibiting activity was obtained. This was followed by 3 rounds of re-cloning to yield a single clone producing a human monoclonal antibody which inhibited labelled TSH binding to the TSH receptor. This human

monoclonal TSH receptor autoantibody was designated hMAb TSHR1 and was of subclass IgG1 with a lambda light chain.

The ability of different concentrations of hMAb TSHR1 IgG and Fab to inhibit labelled TSH binding to the TSH receptor is shown in Figure 1. As can be seen in Figure 1 as little as 1ng/mL of these preparations inhibited TSH binding with more than 90% inhibition being obtained with 1000ng/mL. TSMAb TSHR1 IgG and Fab also stimulated cyclic AMP production in CHO cells transfected with the TSH receptor as shown in Figure 2. As little as 1ng/mL of hMAb TSHR1 IgG or Fab caused strong stimulation of cyclic AMP. Similar levels of stimulation were observed with 0.1ng/mL porcine TSH and 10ng/mL of human TSH. Comparison of the ability of the serum from the original lymphocyte donor (taken at the same time as the blood sample for lymphocyte isolation) to inhibit labelled TSH binding to the TSH receptor and to stimulate cyclic AMP production in TSH receptor transfected CHO cells is shown in Figure 3. Inhibition of TSH binding could be detected with serum diluted 500x whereas stimulation of cyclic AMP could be detected with serum diluted 5000x.

^{125}I-labelled hMAb TSHR1 IgG bound to TSH receptor coated tubes and Scatchard analysis indicated an association constant of 5×10^{10} molar^{-1}. This binding was inhibited by sera from patients with Graves' disease who had TSH receptor autoantibodies (detectable by inhibition of labelled TSH binding) (Table 1). ^{125}I-labelled hMAb TSHR1 Fab also bound to TSH receptor coated tubes (association constant by Scatchard analysis = 4.5×10^{10} molar^{-1}) and this binding was inhibited by TSH receptor autoantibody positive Graves' sera (Table 2). In addition, detergent solubilised preparations were able to bind to plastic tubes coated with hMAb TSHR1 and this binding could be inhibited by sera containing TSH receptor autoantibodies (Table 3).

As shown in Table 4 hMAb TSHR1-biotin bound to TSH receptor coated ELISA plates and the binding was inhibited by the international reference preparation NIBSC 90/672 and serum from patients with Graves' disease. Inhibition of binding was not observed by sera from healthy blood donors. Figure 3a shows a graphical representation of a comparison between an assay for TSHR autoantibodies based on hMAb TSHR1-biotin and earlier assays. The sensitivity of the assay based on hMAb

TSHR1-biotin is clearly superior according to concentration of the international standard NIBSC 90/672 detectable. This was confirmed in a study of sera from 72 patients with Graves' disease shown in Figure 3b. Healthy blood donor sera (n = 100) and sera from subjects with non-thyroid diseases (n = 43) gave respectively values of up to 10% inhibition of hMAb TSHR1 binding and up to 11% inhibition of TSH binding in this study.

hMAb TSHR1 IgG did not react with full length TSH receptor preparations on Western blot analysis nor did it react well with ^{35}S-labelled full length TSH receptor in the immunoprecipitation assay nor in the TSH receptor peptide ELISA. This lack of reactivity indicates that hMAb TSHR1 reacts with conformational rather than linear epitopes on the TSH receptor.

Sequence analysis of the genes coding for hMAb TSHR1 indicated that the heavy chain V region genes were of the VH5 family, the D gene of the D6-13 family and the J gene of the JH5 family and for the light chain the V-gene region is from the VL1-11 germline and the J-gene region is from the JL3b germline. The heavy chain nucleotide and amino acid sequences are shown in Figures 4 and 5 respectively and the light chain nucleotide and amino acid sequences are shown in Figures 6 and 7 respectively. These sequences are a refinement of the HC and LC nucleotide sequences determined using PCR primers which are degenerate. In particular a HC sequencing artefact for nucleotides 115-120 was identified. Sequencing indicated cacgtg (transcribed to amino acids His Val) whereas the crystal structure more reliably indicated amino acids Gln Leu (corresponding bases being cagctg). Crystal structure analysis also enabled refinement of the HC and LC derived amino acid sequences particularly in the degenerate PCR primer region. In the case of the LC amino acid 2 was found to be Pro by RT-PCR but was Thr from the crystal structure. In the case of the HC amino acid 2 was found to be Met by RT-PCR but was Val from the crystal structure.

Comparison of the activities of hMAb TSHR1 IgG preparations and the international standard for TSH receptor autoantibodies in terms of inhibition of labelled TSH binding are shown in Table 5. This enabled a specific activity of hMAb TSHR1 IgG to be estimated as 138 units of NIBSC 90/672 per mg of protein when the assays were

carried out in serum and 163 units per mg when the assays were carried out in assay buffer (mean of the 2 values = 150 units/mg). hMAb TSHR1 Fab preparations were 288 and 309 units per mg in serum and assay buffer respectively (mean of the 2 values = 300 units/mg). Table 6 shows a similar analysis of the lymphocyte donor serum and the donor serum IgG. As can be seen the donor serum contains a mean of 0.38 units/mL of NIBSC 90/672 (0.36 and 0.4 in serum and assay buffer respectively) and the donor serum IgG has a mean specific activity of 0.059 units per mg of protein. These results are summarised in Table 7 and comparison with the specific activity of hMAb TSHR1 IgG (150 units/mg) indicates that the monoclonal autoantibody IgG is 2500 times more active than the lymphocyte donor serum IgG in terms of inhibition of TSH binding.

Initial assessment of the activities of the various IgG and serum preparations in terms of stimulation of cyclic AMP in CHO cells transfected with the TSH receptor are also shown in Table 7. The stimulation of cyclic AMP assay is characterized by considerable within assay and between assay variability. This relates to several factors including variation in the number and quality of cells initially seeded into the 96 well plates and variation in growth rate of the seeded cells over the subsequent 48 hours. Consequently the assays of hMAb TSHR1 IgG and Fab, lymphocyte donor serum and serum IgG and NIBSC 90/672 were carried out repeatedly and the results are summarized in Table 8. The specific activity of the hMAb TSHR1 IgG was 318 units per mg in the stimulation assay compared with 0.1 units per mg for the lymphocyte donor serum IgG ie the monoclonal autoantibody IgG was about 3000 times as active as the donor serum IgG in terms of stimulation of cyclic AMP production. This value is in reasonable agreement with the value of 2500 times observed for inhibition of TSH binding measurements (see above and Tables 5 and 6). Table 9 shows a further analysis of the TSH receptor stimulating effects of hMAb TSHR1 IgG and Fab and the lymphocyte donor serum IgG.

The effects of porcine TSH and hMAb TSHR1 IgG on stimulation of cyclic AMP production in CHO cells expressing the TSH receptor were additive as can be seen in the results shown in Table 10.

Typical results observed in the stimulation of cyclic AMP assay with the reference preparation NIBSC 90/672 are shown in Table 11.

Tables 12 and 13 show the effects of the various *E.coli* culture supernatants in terms of inhibition of labelled TSH binding and stimulation of cyclic AMP production respectively. Transformed (with hMAb THSR1 plasmid) and IPTG induced cultures of both strains of *E.coli* produced sufficient amounts of recombinant hMAb TSHR Fab to act as potent inhibitors of TSH binding (Table 12) and powerful stimulators of cyclic AMP production (Table 13). Control culture supernatants (from untransformed cells and transformed but non-induced cells) did not produce detectable levels of binding inhibition (Table 12) or stimulating (Table 13) activities.

In further control experiments, a recombinant human antibody Fab produced by cloning and expression of the HC and LC of a human monoclonal antibody to GAD (4B4) were analysed. Assays of culture supernatants and periplasmic fractions indicated that recombinant 4B4 Fab did not have detectable inhibition of TSH binding activity (Tables 14 and 15) or TSHR stimulating activity (Tables 16 and 17). Furthermore, hybrid Fabs consisting of (a) hMAb TSHR1 HC and 4B4 LC (b) hMAb TSHR1 LC and 4B4 HC did not show interaction with the TSHR in either assay (Tables 14-17). Assays for GAD Ab activity in these various recombinant Fab preparations were only able to detect GAD Ab expression in cells transformed with 4B4 HC and 4B4 LC (Tables 18 and 19). Recombinant hMAb TSHR1 Fab did not show detectable GAD Ab activity and neither did recombinant Fab hybrids consisting of mixtures of 4B4 and hMAb TSHR1 HC and LC (Tables 18 and 19).

The ability of hMAb TSHR1 to stimulate cyclic AMP production in CHO cells transfected with the TSHR was inhibited by patient sera containing TSHR autoantibodies which acted as TSH antagonists (Figure 8). In addition, a mouse monoclonal antibody to the TSHR (9D33) was able to block hMAb TSHR1 stimulating activities (Table 20) whereas another TSHR mouse MAb (2B4) was ineffective (Table 20). However 2B4 was able to block the stimulating activities of TSH as was 9D33 (Table 21). Table 22 shows the ability of 2B4 and 9D33 to inhibit the binding of ^{125}I-labelled TSH and ^{125}I-labelled hMAb TSHR1 to TSHR plastic tubes. 9D33 was able to inhibit labelled TSH binding and labelled hMAb TSHR1

binding quite effectively (more than 50% inhibition at 10 μg/mL). 2B4 was an effective inhibitor of labelled TSH binding to the TSHR (more than 80% inhibition at 1 μg/mL) but had only a minor effect on hMAb TSHR1 binding (11% inhibition at 1 μg/mL) or on 9D33 binding (22% inhibition at 1 μg/mL). The binding of labelled 9D33 to TSHR coated tubes was inhibited by sera from patients with Graves' disease containing TSHR autoantibodies (as measured by inhibition of labelled TSH binding to the TSHR) whereas healthy blood donor sera and sera from patients with other autoimmune diseases had little or no effect (Table 23). Labelled 9D33 binding to the TSHR was inhibited by TSHR autoantibodies with TSH agonist or antagonist properties (Table 24) and by the international standard NIBSC 90/672 (Table 25). Scatchard analysis indicated that 9D33 and 2B4 had affinities of 2×10^{10} $molar^{-1}$ and 1×10^{10} $molar^{-1}$ respectively for TSH receptors coated onto plastic tubes.

Immunisation of mice with hMAb TSHR1 Fab resulted in production of antibodies in the mice sera (polyclonal antibodies) which were capable of binding to hMAb TSHR1 Fab in such a way as to inhibit the ability of the Fab to bind to the TSHR (Table 26). Furthermore, a monoclonal antibody produced from the spleen cells of a mouse immunised with hMAb TSHR1 Fab was also able to inhibit the Fab binding to the TSHR (Table 27).

Overall our analysis indicates that the human monoclonal autoantibody hMAb TSHR1 has the TSH receptor binding and thyroid stimulating characteristics of the TSH receptor autoantibodies in the serum of the lymphocyte donor. As detailed above, the monoclonal antibody was also produced as a recombinant Fab preparation.

CONCLUSIONS

(a) We have produced a human monoclonal autoantibody to the TSH receptor which has similar properties to the TSH receptor autoantibody in the donor patient's serum. The monoclonal antibody was also produced as a recombinant Fab preparation.

(b) The monoclonal antibody IgG and Fab and recombinant Fab preparations are powerful thyroid stimulators and effective inhibitors of labelled TSH binding to the TSH receptor.

(c) Binding of labelled MAb IgG and Fab preparations to the TSH receptor is inhibited by TSH receptor autoantibody positive sera from patients with Graves' disease but not by healthy blood donor sera or sera from patients with other autoimmune diseases. Assay systems for TSHR autoantibodies based on inhibition of labelled hMAb TSHR1 binding to the TSH receptor are more sensitive than other assays so far described.

(d) TSH receptor autoantibodies which act as TSH antagonists as well as TSH receptor autoantibodies which act as TSH agonists inhibit labelled hMAb TSHR1 binding to the TSH receptor.

(e) hMAb TSHR1 preparations coated onto plastic tubes bind TSH receptor and this binding is inhibited by TSH receptor autoantibodies in different patient sera.

(f) A mouse monoclonal antibody (9D33) which inhibits hMAb TSHR1 binding to the TSHR was also found to block the stimulating activity of hMAb TSHR1 and TSH.

(g) Mouse polyclonal and monclonal antibodies to hMAb TSHR1 have been produced which bind to hMAb TSHR1 in such a way as to prevent it binding to the TSH receptor. These anti-idiotypic antibodies compete therefore with the TSHR for hMAb TSHR1 and as such they may be useful alternatives to the TSHR in applications where a binding partner for TSHR autoantibodies is required.

(h) These results indicate that hMAb TSHR1 and/or its derivatives and/or its competitors can be used as a replacement for TSH in

(i) assays for TSH receptor autoantibodies, TSH and related ligands

(ii) various in vivo applications involving provision of TSH agonist or TSH antagonist activities.

(iii) identification and provision of new types of TSH receptor autoantibody binding sites.

Table 1 Effect of patient sera on ^{125}I-labelled hMAb TSHR1 IgG binding to the TSH receptor and comparison with effect on ^{125}I-labelled TSH binding to the TSH receptor

Test material	inhibition of labelled hMAb TSHR1 binding	inhibition of TSH binding	Test material	inhibition of labelled hMAb TSHR1 binding	inhibition of TSH binding
G1	62	80	N1	3.1	7.7
G2	91	93	N2	2.4	2.6
G3	91	76	N3	-1.0	4.5
G4	94	92	N4	-11	6.5
G5	93	94	N5	1.7	5.0
G6	76	85	N6	2.8	1.7
G7	87	90	N7	5.2	-0.8
G8	65	45	N8	3.5	0.2
G9	88	90	N9	2.8	-0.6
G10 /10	83	59	N10	4.5	2.2
G10 /20	69	43	D1	-4.8	2.2
G10 /40	56	29	A1	-3.1	1.3
G10 /80	42	19	A2	-3.5	-3.0
G11 /10	75	73			
G11 /20	59	54			
G11 /40	39	33			
G11 /80	22	18			

G1-G11 are sera from patients with a history of Graves' disease.

G9 serum has high levels of TSH blocking (ie TSH antagonist activity).

G10 and G11 have high levels of thyroid stimulating activity.

G10 is the lymphocyte donor serum.

/10, /20 etc indicate dilution factor in a pool of healthy blood donor sera.

N1-N10 are sera from healthy blood donors.

D1 is from a patient with type 1 diabetes mellitus (positive for autoantibodies to glutamic acid decarboxylase).

A1 and A2 are from patients with Addison's disease (positive for steroid 21-hydroxylase autoantibodies).

In the presence of the pool of healthy blood donor sera about 25% of ^{125}I-labelled MAb IgG bound to the TSHR coated tubes.

Table 2 Effect of patient sera on ^{125}I-labelled hMAb TSHR1 Fab binding to the TSH receptor and comparison with effect on ^{125}I-labelled TSH binding to the TSH receptor

Test material	inhibition of labelled Fab binding	inhibition of TSH binding
NIBSC 90/672 diluted in a pool of healthy blood donor serum		
to 1 U/L	17	13
to 2 U/L	27	24
to 4 U/L	47	44
to 8 U/L	61	65
Healthy blood donor serum A	-3	<10
Healthy blood donor serum B	3	<10
Healthy blood donor serum C	4	<10
Healthy blood donor serum D	-4	<10
Healthy blood donor serum E	0	<10
Graves' serum F	64	78
Graves' serum G	42	54
Graves' serum H	49	69
Graves' serum I	24	36
Graves' serum J	76	88

Table 3 Binding of TSHR to plastic tubes coated with hMAb TSHR 1 Fab and inhibition of TSHR binding by sera containing TSHR autoantibodies

Test material	cpm bound[1]
Healthy blood donor serum A	8406
Healthy blood donor serum B	8430
TSHR autoantibody positive serum 1	1527
TSHR autoantibody positive serum 2	1131
TSHR autoantibody positive serum 3	1199

[1] TSHR binding was detected using a ^{125}I-labelled mouse monoclonal antibody to the TSHR C terminus; total cpm = 39,000 per tube.

Table 4 Effect of patient serum samples on binding of biotin labelled hMAb TSHR1 and biotin labelled TSH to ELISA plate wells coated with TSHR

	hMAb TSHR1 biotin		TSH biotin	
	OD_{450}	% inhibition	OD_{450}	% inhibition
HBD pool	1.852	0	1.778	0
HBD pool plus 1U/mL	1.46	21	1.489	16
HBD pool plus 2U/mL	1.168	37	1.304	27
HBD pool plus 4U/mL	0.792	57	0.947	47
HBD pool plus 8U/mL	0.539	71	0.492	72
HBD pool plus 40U/mL	0.118	94	0.233	87
Serum P1	1.415	24	1.397	21
Serum P2	1.264	32	1.256	29
Serum P3	0.558	70	0.408	77
Serum P4	0.763	59	0.907	49
Serum P5	1.047	43	-	
Serum P6	0.843	55	-	
Serum P7	1.429	23	-	
HBD 1	1.745	6	1.713	4
HBD 2	1.807	2	-	
HBD 3	1.779	4	1.626	9
HBD 4	1.821	2	-	
HBD 5	1.841	1	1.660	7
HBD 6	1.762	5	1.777	0
HBD 7	1.799	3	1.767	1
HBD 8	1.783	4	1.703	4
HBD 9	1.792	3	1.669	3

HBD = healthy blood donor serum

U/mL are units of NIBSC 90/672

Serum P1-P7 are from patients with Graves' disease

Table 5 Inhibition of TSH binding by WHO reference preparation NIBSC 90/672 and by hMAb TSHR1 IgG and Fab preparations

Sample	Samples diluted in serum[1]				Samples diluted in assay buffer			
	% inhibition	units/L	units/mg	mean units/mg	% inhibition	units/L	units/mg	mean units/mg
NIBSC 90/672								
0.125 units/L					2			
0.25 units/L					4			
0.5 units/L					11			
1.0 units/L	15				19			
2.0 units/L	28				38			
4.0 units/L	48				64			
8.0 units/L	69				83			
40.0 units/L	95				94			
hMAb TSHR1 IgG								
0 ng/mL	1				0			
0.3 ng/mL	1				2			
1 ng/mL	3				3			
3 ng/mL	7				10	0.46		
10 ng/mL	21	1.48	148		33	1.73	173	
30 ng/mL	46	3.9	130	138	70	4.8	160	163
100 ng/mL	81	13.5	135		92	15.6	156	
300 ng/mL	92				95	>40		
hMAb TSHR1 Fab								
0.3 ng/mL	5				-2			
1 ng/mL	5				1			
3 ng/mL	16	1.05	351		16	0.8	265	
10 ng/mL	36	2.77	277		52	2.9	291	309
30 ng/mL	69	8.0	267	288	86	9.6	372	
100 ng/mL	89	23.7	237		92	16.9		
300 ng/mL	93				94			
2G4 IgG[2]								
0.3 ng/mL	2				-3			
3 ng/mL	1				-6			
30 ng/mL	0				-5			
300 ng/mL	3				-4			
2G4 Fab[2]								
0.3 ng/mL	4				-5			
3 ng/mL	4				-6			
30 ng/mL	1				-5			
300 ng/mL	2				-6			

[1] Pool of healthy blood donor serum, 14.9% of total cpm bound to the TSHR in the presence of this serum pool only. 14.7% of total cpm bound to the TSHR in the presence of buffer only.

[2] 2G4 is a human monoclonal autoantibody to thyroid peroxidase.

Table 6 Inhibition of TSH binding by lymphocyte donor serum and donor serum IgG

Sample	Samples diluted in serum[1]				Samples diluted in assay buffer			
	% inhibition	units/L[2]	units/mg or (units/mL in undiluted serum)	mean units/mg or (units/mL)	% inhibition	units/L[2]	units/mg or (units/mL in undiluted serum)	mean units/mg or (units/mL)
Donor serum								
diluted 1000x	6				10			
diluted 300x	18	1.2	(0.36)		28	1.3	(0.39)	
diluted 100x	42	3.2	(0.32)	(0.36)	62	3.9	(0.39)	(0.40)
diluted 30x	78	11.3	(0.39)		91	13.5	(0.41)	
diluted 10x	93	34			95	>40		
Donor serum IgG								
0 mg/mL	0	0			0			
0.01 mg/mL	7				19	0.87		
0.03 mg/mL	23	1.6	0.053		37	1.9	0.063	
0.1 mg/mL	57	5.1	0.051	0.054	78	6.4	0.064	0.063
0.3 mg/mL	85	17	0.057		93	19	0.063	
1 mg/mL	96	43			96	>40		
Healthy blood donor pool serum								
diluted 1000x	0				3			
diluted 100x	1				4			
diluted 10x	1				11			
Healthy blood donor pool serum IgG								
0.01 mg/mL	2				2			
0.1 mg/mL	1				5			
1 mg/mL	3				7			

[1] Pool of healthy blood donor serum, 14.7% of total cpm bound to the TSHR in the presence of this serum pool only. 16.3% of total cpm bound to the TSHR in the presence of buffer only.

[2] Units shown are NIBSC 90/672 international TSHR autoantibody reference preparation.

Table 7 Specific activities of hMAb TSHR1 and lymphocyte donor serum and IgG preparations

	Inhibition of TSH binding assay		Stimulation of cyclic AMP assay	
Preparation	**Units/mg[1,2]**	**Units/nmole[1,2]**	**Units/mg[1]**	**Units/nmole[1]**
hMAb TSHR1 IgG	150	22	180	26
hMAb TSHR1 Fab	300	15	700	35
Donor serum IgG	0.059	0.009	0.33	0.048
Donor serum units/mL	0.38		1.8	

[1] Units shown are NIBSC 90/672.
[2] Values are a mean of results obtained in serum and in assay buffer (see Tables 5 and 6)

Table 8 Summary of specific activities of hMAb TSHR1 and lymphocyte donor serum and serum IgG determined in several stimulation of cyclic AMP assays

Preparation	Mean Units per mg	Number of determinations	Standard Deviation
hMAb TSHR1 IgG	318	16	189
hMAb TSHR1 Fab	492	10	184
Donor serum IgG	0.10	10	0.08
Donor serum units/mL	0.9	4	0.6

Table 9 Further analysis of the effects of hMAb TSHR1 IgG and Fab and lymphocyte donor serum IgG in the cyclic AMP stimulation assay

Sample	Mean cyclic AMP per well (pmols)	number of determinations	Standard Deviation
hMAb TSHR1 IgG			
0ng/mL	0.96	6	0.048
0.3ng/mL	1.25	6	0.12
1ng/mL	1.84	6	0.16
3ng/mL	3.4	5	0.37
10ng/mL	6.6	5	0.62
hMAb TSHR1 Fab			
0ng/mL	0.60	6	0.068
0.3ng/mL	1.11	6	0.11
1ng/mL	1.99	6	0.39
3ng/mL	4.9	6	0.44
10ng/mL	10.6	6	0.86
Control human MAb (2G4)[1]			
IgG 0ng/mL	0.72	11	0.19
IgG 10ng/mL	0.61	11	0.16
Fab 10ng/mL	0.61	4	0.044
Lymphocyte donor serum IgG			
3µg/mL	1.67	6	0.38
10µg/mL	4.20	6	0.93
30µg/mL	6.22	6	0.73
Healthy blood donor pool serum IgG			

30μg/mL	0.38	6	0.10

[1] 2G4 is a human monoclonal autoantibody to thyroid peroxidase

Table 10 Additive effect of TSH and hMAb TSHR1 IgG in stimulation of cyclic AMP assays

Experiment 1			Experiment 2		
	Sample	cyclic AMP[1] (pmols per well)		Sample	cyclic AMP[1] (pmols per well)
A	Buffer only	0.57	A	Buffer only	0.42
B	Porcine TSH 0.1ng/mL	1.07	B	Porcine TSH 0.05ng/mL	1.07
C	hMAb TSHR1 1ng/mL	1.41	C	hMAb TSHR1 0.5ng/mL	0.92
B plus C		2.08	B plus C		1.92

[1] Values shown are means of closely agreeing duplicate determinations

Table 11 Effects of NIBSC 90/672 in the cyclic AMP stimulation assay

Sample	Mean cyclic AMP per well (pmols)	number of determinations	Standard Deviation
Buffer only	0.60	6	0.068
0.1 units/L	1.09	6	0.085
0.3 units/L	1.49	5	0.11
1.0 units/L	3.52	5	0.46
3.0 units/L	8.16	6	1.39

Table 12 **Inhibition of ^{125}I-TSH binding to TSHR coated tubes by recombinant hMAb TSHR1 Fab expressed in 2 different *E coli* strains (XL1-Blue MRF' and HB2151 cells)**

Sample	Culture supernatant dilution[2]	% binding	% inhibition[3]
Assay buffer only[1]		12.1	0
Untransformed XL1-Blue MRF' cell culture supernatant	4x	11.6	3.9
	8x	11.5	4.5
	16x	11.9	1.5
	32x	11.9	1.5
	64x	11.9	1.0
	128x	12.1	-0.5
Transformed but non-induced XL1-Blue MRF' cell culture supernatant	4x	11.5	5.0
	8x	11.6	4.2
	16x	11.8	2.2
	32x	11.1	8.4
	64x	11.7	3.4
	128x	11.1	8.0
Transformed and induced XL1-Blue MRF' cell culture supernatant	4x	1.5	87.4
	8x	2.3	81.3
	16x	4.7	60.9
	32x	7.2	40.2
	64x	8.9	26.4
	128x	10.3	14.8
Untransformed HB2151 cell culture supernatant	x4	11.2	7.3
	x8	11.1	8.1
	x16	10.9	9.7
	x32	10.8	10.2
	x64	10.8	10.2
	x128	10.6	12.3
Transformed but non-induced HB2151 cell culture supernatant	4x	10.7	11.6
	8x	10.5	13.3
	16x	10.6	11.8
	32x	10.7	11.4
	64x	10.9	9.6
	128x	10.6	11.8
Transformed and induced HB2151 cell culture supernatant	4x	1.0	92.0
	8x	1.0	91.4
	16x	1.3	89.0
	32x	2.2	82.0
	64x	4.3	64.1
	128x	6.7	44.8

[1]Assay buffer = 50mmol/L NaCl, 10mmol/L Tris-HCl pH 7.8
[2]All dilutions were in assay buffer
[3] Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left\{ \frac{A}{B} \times 100 \right\}$$

where A = binding in the presence of test sample
B = binding in the presence of assay buffer

Table 13 **Stimulation of cAMP production in CHO cells transfected with the TSHR by recombinant hMAb TSHR1 Fab expressed in 2 different *E coli* strains (XL1-Blue MRF' and HB2151 cells)**

Sample	Culture supernatant dilution[2]	pmol/cell well	mean	x basal[3]
Assay buffer only[1]		0.54	0.49	1
		0.44		
Untransformed XL1-Blue MRF' cell culture supernatant	10x	0.32	0.33	0.68
		0.35		
Transformed but non-induced XL1-Blue MRF' cell culture supernatant	10x	0.52	0.62	1.3
		0.73		
	50x	0.50	0.49	0.99
		0.48		
Transformed and induced XL1-Blue MRF' cell culture supernatant	10x	>6.4	>6.4	>13.1
		>6.4		
	50x	3.5	3.6	7.3
		3.6		
Untransformed HB2151 cell culture supernatant	10x	0.39	0.37	0.76
		0.35		
Transformed but non-induced HB2151 cell culture supernatant	10x	0.29	0.37	0.76
		0.45		
	50x	0.37	0.37	0.76
		0.37		
Transformed and induced HB2151 cell culture supernatant	10x	>6.4	>6.4	>13.1
		>6.4		
	50x	>6.4	>6.4	>13.1
		>6.4		

[1]Assay buffer = Hanks' buffered salt solution (NaCl free) containing 1 g/L glucose, 20 mmol/L HEPES, 222 mmol/L sucrose, 15 g/L bovine serum albumin (BSA) and 0.5 mmol/L 2 isobutyl-1-methyl xanthine pH 7.4

[2] Dilutions in assay buffer

[3] Basal = cAMP produced in the presence of assay buffer only

Table 14 Inhibition of ^{125}I-TSH binding to TSHR coated tubes by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab (a human MAb to GAD) and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151 cells

ASSAYS OF PERIPLASMIC FRACTIONS

Test sample	Periplasmic fraction (PF) dilution and (total Fab concentration in undiluted PF)	% ^{125}I-TSH binding	% inhibition[1]
Assay buffer only		11.5	0
Untransformed cells	4x	11.8	-2.7
	8x (ud)	11.8	-2.7
	16x	12.2	-5.9
hMAb TSHR1 HC/LC transformed but non-induced cells	4x	1.6	86[a]
	8x (177 ng/mL)	3.6	68
	16x	6.4	45
hMAb TSHR1 HC/LC transformed and induced cells	4x	0.95	92
	8x (364 ng/mL)	1.4	88
	16x	2.7	77
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	4x	12.0	-3.9
	8x (ud)	12.1	-4.8
	16x	12.4	-7.8
hMAb TSHR1 HC/4B4 LC transformed and induced cells	4x	11.4	0.9
	8x (83 ng/mL)	11.8	-2.1
	16x	11.7	-1.8
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	4x	11.9	-3.5
	8x (ud)	12.2	-6.1
	16x	12.4	-7.8
4B4 HC/hMAb TSHR1 LC transformed and induced cells	4x	11.8	-2.8
	8x (850 ng/mL)	11.2	3.1
	16x	11.9	-3.2
4B4 HC/LC transformed but non induced cells	4x	12.1	-5.4
	8x (ud)	12.0	-4.0
	16x	12.1	-4.8
4B4 HC/LC transformed and induced cells	4x	11.7	-1.2
	8x (265 ng/mL)	11.7	-1.4
	16x	12.0	-4.2

[1]Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{A}{B} \times 100\right]$$

where A = % ^{125}I-TSH binding in the presence of test sample
B = % ^{125}I-TSH binding in the presence of assay buffer

[a]Detection of TSHR Ab activity in the non-induced cells was due to constitutive activity of the promoter giving low level expression of Fab in the absence of IPTG.

ud = undetectable

Table 15 Inhibition of ^{125}I-TSH binding to TSHR coated tubes by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab (a human MAb to GAD) and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151 cells

ASSAYS OF CULTURE SUPERNATANTS

Test sample	Culture supernatant dilution and (total Fab concentration in undiluted supernatant)	% ^{125}I-TSH binding	% inhibition[1]
Assay buffer only		11.1	0
Untransformed cells	4x	11.8	-6.5
	8x (ud)	13.1	-18
	16x	11.1	-0.3
hMAb TSHR1 HC/LC transformed but non-induced cells	4x	10.8	2.1
	8x (ud)	12.1	-9.8
	16x	11.9	-8.1
hMAb TSHR1 HC/LC transformed and induced cells	4x	1.1	91
	8x (421 ng/mL)	1.2	90
	16x	1.1	90
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	4x	11.8	-7.0
	8x (ud)	12.5	-13.0
	16x	12.0	-1.3
hMAb TSHR1 HC/4B4 LC transformed and induced cells	4x	10.8	2.7
	8x (262 ng/mL)	11.1	-0.4
	16x	11.3	-2.6
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	4x	11.9	-7.4
	8x (ud)	12.7	-15
	16x	11.8	-7.0
4B4 HC/hMAb TSHR1 LC transformed and induced cells	4x	10.5	4.8
	8x (84 ng/mL)	10.8	2.4
	16x	11.2	-0.9
4B4 HC/LC transformed but non-induced cells	4x	11.9	-7.5
	8x (ud)	12.6	-14
	16x	12.0	-9.0
4B4 HC/LC transformed and induced cells	4x	10.5	-4.7
	8x (522 ng/mL)	11.0	0.7
	16x	11.0	0.5

[1]see footnote to Table 14

ud = undetectable

Table 16 Stimulation of cyclic AMP production in CHO Cells transfected with the TSHR by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab (a human MAb to GAD) and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151 cells

ASSAYS OF CULTURE SUPERNATANTS

Test sample[1]	Dilution[2] of culture supernatant and (total Fab concentration in undiluted supernatants)	pmol cyclic AMP /cell well	mean pmol cyclic AMP /cell well	x basal stimulation[3]
Assay buffer[1] only		0.35 0.25 0.33	0.31	1
Untransformed cells	10x (ud)	0.51 0.67 0.48	0.55	1.8
hMAb TSHR1 HC/LC transformed but non-induced cells	10x (ud)	0.84 1.80 2.13	1.59	5.1[a]
hMAb TSHR1 HC/LC transformed and induced cells	10x (421 ng/mL)	>6.4 >6.4 >6.4	>6.4	>20
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	10x (ud)	0.55 0.63 0.58	0.59	1.9
hMAb TSHR1 HC/4B4 LC transformed and induced cells	10x (262 ng/mL)	0.47 0.47 0.52	0.48	1.6
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	10x (ud)	0.65 0.59 0.60	0.61	2.0
4B4 HC/hMAb TSHR1 LC transformed and induced cells	10x (84 ng/mL)	0.51 0.37 0.38	0.42	1.4
4B4 HC/LC transformed but non induced cells	10x (ud)	0.65 0.73 0.64	0.67	2.2
4B4 HC/LC transformed and induced cells	10x (522 ng/mL)	0.55 0.41 0.35	0.44	1.4

[1]Assay buffer: Hanks' buffered salt solution (NaCl free) containing 1 g/L glucose, 20 mmol/L HEPES, 222 mmol/L sucrose, 15 g/L bovine serum albumin (BSA) and 0.5 mmol/L 2 isobutyl-1-methyl xanthine pH 7.4

[2]Dilutions in assay buffer

[3]Basal = cyclic AMP production in the presence of assay buffer only

[a]Detection of cyclic AMP stimulation activity in the non-induced cells was due to constitutive activity of the promoter giving low level expression of Fab in the absence of IPTG. Total recombinant Fab levels were undetectable (detection limit = 5-10 ng/mL) whereas the stimulation of cyclic AMP assay can detect as little as 0.3 ng/mL of hMAb TSHR1 Fab.

ud = undetectable

Table 17 Stimulation of cyclic AMP production in CHO Cells transfected with the TSHR by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab (a human MAb to GAD) and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151 cells

ASSAYS OF PERIPLASMIC FRACTIONS

Test sample	Periplasmic fraction (PF) dilution[2] and (total Fab concentration in undiluted PF)	pmol cyclic AMP /cell well	mean pmol cyclic AMP /cell well	x basal stimulation[3]
Assay buffer[1] only		0.35 0.25 0.33	0.31	1
Untransformed cells	10x (ud)	0.31 0.22 0.35	0.29	0.9
hMAb TSHR1 HC/LC transformed but non-induced cells	10x (177 ng/mL)	>6.4 >6.4 >6.4	>6.4	>20.6[a]
hMAb TSHR1 HC/LC transformed and induced cells	10x (364 ng/mL)	>6.4 >6.4 –	>6.4	>20.6
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	10x (ud)	0.40 0.33 0.33	0.35	1.1
hMAb TSHR1 HC/4B4 LC transformed and induced cells	10x (83 ng/mL)	0.31 0.31 0.41	0.34	1.1
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	10x (ud)	0.29 0.31 0.29	0.30	1.0
4B4 HC/hMAb TSHR1 LC transformed and induced cells	10x (850 ng/mL)	0.23 0.25 0.24	0.24	0.8
4B4 HC/LC transformed but non induced cells	10x (ud)	0.33 0.35 0.29	0.32	1.0
4B4 HC/LC transformed and induced cells	10x (265 ng/mL)	0.40 0.38 0.32	0.37	1.2
hMAb TSHR1 IgG 1ng/ml (hybridoma produced)		2.0 2.0 2.0	2.0	6.4

ud = undetectable; [1,2,3]see footnote to Table 16

[a]Detection of TSHR Ab activity in the non-induced cells was due to constitutive activity of the promoter giving low level expression in the absence of IPTG.

Table 18 Inhibition of 4B4 IgG (a hybridoma produced human MAb to GAD) binding to ^{125}I-GAD by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151 cells

ASSAYS OF PERIPLASMIC FRACTIONS

Test sample added with 4B4 IgG and ^{125}I-GAD	Periplasmic fraction (PF) dilution and (total Fab concentration in undiluted PF)	% ^{125}I-GAD binding	% inhibition[1]
GAD Ab Assay buffer		28	0
4B4 F(ab')$_2$ (hybridoma produced) 1μg/ml		5.5	80
0.1μg/ml		12	57
0.01μg/ml		24	14
0.001μg/ml		29	-3.9
Untransformed cells	4x	27	0.9
	8x (ud)	28	-1.7
	16x	29	-6.3
hMAb TSHR1 HC/LC transformed but non-induced cells	4x	28	-2.6
	8x (177 ng/mL)	28	-0.5
	16x	28	-0.8
hMAb TSHR1 HC/LC transformed and induced cells	4x	27	0.7
	8x (364 ng/mL)	27	1.4
	16x	29	-4.2
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	4x	28	-1.1
	8x (ud)	28	-0.2
	16x	27	1.1
hMAb TSHR1 HC/4B4 LC transformed and induced cells	4x	28	-1.4
	8x (83 ng/mL)	28	-0.7
	16x	28	-2.4
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	4x	28	-2.9
	8x (ud)	28	-3.2
	16x	28	-3.1
4B4 HC/hMAb TSHR1 LC transformed and induced cells	4x	27	1.7
	8x (850 ng/mL)	28	-0.2
	16x	28	-1.0
4B4 HC/LC transformed but non induced cells	4x	29	-4.4
	8x (ud)	28	-1.5
	16x	27	1.7
4B4 HC/LC transformed and induced cells	4x	21	23.2
	8x (265 ng/mL)	24	12.5
	16x	26	5.6

[1]Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{A}{B} \times 100\right]$$

where A = ^{125}I-GAD binding in the presence of test sample
B = ^{125}I-GAD binding in the presence of assay buffer

ud = undetectable

Table 19 Inhibition of 4B4 IgG (a hybridoma produced human MAb to GAD) binding to ^{125}I-GAD by recombinant hMAb TSHR1 Fab, recombinant 4B4 Fab and recombinant hybrid Fabs (mixed HC and LC of the 2 Fabs). Expression in *E coli* HB2151

ASSAYS OF CULTURE SUPERNATANTS

Test sample added with 4B4 IgG and ^{125}I-GAD	Culture supernatant dilution and (total Fab concentration in undiluted supernatant)	% ^{125}I-GAD binding	% inhibition[1]
Assay buffer		26	0
4B4 F(ab')$_2$ (hybridoma produced) 1μg/ml		5.2	80
0.1μg/ml		11	58
0.01μg/ml		22	15
0.001μg/ml		28	-5.2
Untransformed HB2151 cells	4x	28	-5.5
	8x (ud)	29	-9.1
	16x	28	-6.3
hMAb TSHR1 HC/LC transformed but non-induced cells	4x	28	-7.0
	8x (ud)	29	-9.5
	16x	28	-5.2
hMAb TSHR1 HC/LC transformed and induced cells	4x	28	-7.0
	8x (421 ng/mL)	28	-6.4
	16x	28	-5.6
hMAb TSHR1 HC/4B4 LC transformed but non-induced cells	4x	29	-9.0
	8x (ud)	29	-7.9
	16x	28	-7.6
hMAb TSHR1 HC/4B4 LC transformed and induced cells	4x	27	-2.2
	8x (262 ng/mL)	28	-5.5
	16x	28	-5.1
4B4 HC/hMAb TSHR1 LC transformed but non-induced cells	4x	28	-4.4
	8x (ud)	28	-7.0
	16x	28	-5.5
4B4 HC/hMAb TSHR1 LC transformed and induced cells	4x	27	-2.0
	8x (84 ng/mL)	28	-5.7
	16x	27	-2.5
4B4 HC/LC transformed but non induced cells	4x	28	-4.8
	8x (ud)	28	-4.8
	16x	27	-1.7
4B4 HC/LC transformed and induced cells	4x	11	59
	8x (522 ng/mL)	14	46
	16x	17	34

[1]see footnote to Table 18

ud = undetectable

Table 20 Effects of mouse monoclonal antibodies to the TSHR on hMAb TSHR1 induced stimulation of cyclic AMP production in CHO cells expressing the TSHR

Test sample[1]	pmol cyclic AMP/cell well	mean pmol cyclic AMP/cell well	SD	x basal stimulation[2]
hMAb TSHR1 Fab 5 ng/mL plus:-				
(a) Assay buffer[1]	3.252 2.418 –	2.835	–	3.9
(b) 2G2[3]	4.278 3.392 3.116	3.595	0.496	4.9
(c) 2B4[4]	3.320 2.632 2.864	2.939	0.286	4.0
(d) 9D33[4]	0.506 0.394 0.428	0.443	0.047	0.61
Assay buffer alone[1]	0.696 0.742 0.742	0.727	0.02	1
2G2 alone[3]	0.252 0.306 0.376	0.311	0.051	0.43
2B4 alone[4]	0.298 0.318 0.376	0.331	0.033	0.46
9D33 alone[4]	0.280 0.318 0.340	0.313	0.025	0.43

[1]All dilutions were made in assay buffer (Hanks' buffered salt solution (NaCl free) containing 1 g/L glucose, 20 mmol/L HEPES, 222 mmol/L sucrose, 15 g/L bovine serum albumin (BSA) and 0.5 mmol/L 2 isobutyl-1-methyl xanthine pH 7.4)

[2]Basal = cyclic AMP production in the presence of assay buffer only

[3]2G2 is a mouse monoclonal antibody to thyroglobulin (100 μg/mL of IgG preparation)

[4]2B4 and 9D33 are mouse monoclonal antibodies to the TSHR (100 μg/mL of IgG preparations)

Table 21 Effects of mouse monoclonal antibodies to the TSHR on pTSH induced stimulation of cyclic AMP production in CHO cells expressing the TSHR

Test sample[1]	pmol cyclic AMP/cell well	mean pmol cyclic AMP/cell well	SD	x basal stimulation[2]
pTSH 0.5 ng/mL plus:-				
(a) Assay buffer[1]	4.016 2.746 4.960	3.91	0.91	11.5
(b) 2B4[4]	0.878 0.710 0.742	0.78	0.07	2.3
(c) 9D33[4]	0.436 0.436 0.410	0.43	0.01	1.3
Assay buffer alone[1]	0.384 0.318 0.318	0.34	0.03	1
2B4 alone[4]	0.446 0.486 0.552	0.49	0.04	1.4
9D33 alone[4]	0.332 0.362 0.304	0.33	0.02	0.97

[1,2,4]See footnotes for Table 20. In a separate experiment a control mouse MAb to thyroglobulin (2G2 IgG 100 μg/mL) was shown to have no effect on pTSH (0.5 ng/mL) stimulation of cyclic AMP production (pTSH plus assay buffer = 12.7 x basal; pTSH plus 2G2 = 11.7 x basal)

Table 22 Inhibition by various MAbs of ^{125}I-TSH , ^{125}I-hMAb TSHR1 or ^{125}I-9D33 binding to TSHR coated tubes

Test IgG and concentration (μg/mL)[1]		Inhibition of ^{125}I-TSH binding[2]	Inhibition of ^{125}I-hMAb TSHR1 binding[2]	Inhibition of ^{125}I-9D33 binding[2]
9D33 IgG	0.001	0	0	4
	0.01	17	3	9
	0.1	34	21	35
	1	58	44	64
	10	68	56	71
2B4 IgG	0.001	15	0	4
	0.01	36	0	5
	0.1	62	0	15
	1	83	11	22
	10	85	18	22
hMAb TSHR1 IgG	0.001	13	41	1.1
	0.01	60	70	18
	0.1	89	88	72
	1	94	93	90
	10	95	94	93

[1]All dilutions were in a pool of healthy blood donor sera (HBD pool)

[2]Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{A}{B} \times 100\right]$$

where A = % binding in the presence of test sample
B = % binding in the presence of HBD pool

The control mouse MAb to thyroglobulin (2G2 0.001-100 μg/mL) had no effect on the binding of labelled TSH, hMAb TSHR1 or 9D33

Table 23 Effect of patient sera on ^{125}I-9D33 binding and ^{125}I-TSH binding to TSHR coated tubes

Test serum[1]	^{125}I-9D33 bound (% total counts added)	Inhibition of ^{125}I-9D33 binding (%)[2]	Inhibition of ^{125}I-TSH binding (%)[2]
HBD pool	11	0	0
G1	2.2	79	90
G2	4.3	74	59
G3	3.2	69	78
G4	5.8	45	50
G5	4.0	62	78
G6	5.1	67	51
G7	5.9	44	74
G8	2.6	75	82
G9	2.0	81	90
G10	5.5	48	62
G11	3.1	62	59
G12	4.0	43	51
G13	6.0	50	59
G14	5.3	71	80
G15	3.1	77	98
G16	2.4	80	93
G17	2.1	84	94
G18	1.7	73	83
G19	2.9	80	94
G20	2.1	71	80
A1	10	1.9	0
A2	10	2.3	0
D1	11	0	0
D2	10	4.5	0
N1	12	-15	6.7
N2	7.9	25	4.1
N3	11	0	6.3
N4	11	-5.0	6.5
N5	9.1	14	6.3
N6	11	-2.1	7.2
N7	14	-37	-1.4
N8	11	-2.1	5.9
N9	12	-9.8	6.7

[1]HBD pool = pool of healthy blood donor sera
G1-G20 are sera from patients with Graves' disease
D1 and D2 are from patients with type 1 diabetes mellitus (positive for autoantibodies to glutamic acid decarboxylase)
A1 and A2 are from patients with Addison's disease (positive for steroid 21-hydroxylase autoantibodies)
N1-N9 are from individual healthy blood donors

[2]Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{A}{B} \times 100\right]$$

where A = % binding in the presence of test serum
B = % binding in the presence of a pool of healthy blood donor sera

Table 24 Effect of patient sera with TSH agonist and TSH antagonist activities on ^{125}I-9D33 binding to TSHR coated tubes

Test sample and dilution[1]		^{125}I-9D33 bound (% total counts added)	Inhibition of ^{125}I-9D33 binding (%)[2]
HBD pool		11	0
Serum A	1:320	6.4	39
	1:160	4.7	55
	1:80	3.3	69
	1:40	2.8	73
	1:20	2.4	77
	1:10	2.0	82
Serum B	1:320	9.1	13
	1:160	8.3	21
	1:80	6.4	39
	1:40	4.9	53
	1:20	3.8	64
	1:10	2.5	76
Serum C	1:320	9.7	7.6
	1:160	8.9	15
	1:80	8.0	24
	1:40	6.3	40
	1:20	5.1	51
	1:10	3.7	65
Serum D	1:320	9.4	11
	1:160	8.2	22
	1:80	7.2	32
	1:40	5.2	51
	1:20	4.3	59
	1:10	3.3	69

[1]HBD pool = pool of healthy blood donor sera, test sera were diluted in this pool
sera A and B have TSH antagonist activity
sera C and D have TSH agonist activity

[2]Inhibition of binding was calculated with the formula used in Table 23

Table 25 Effect of NIBSC 90/672 on ^{125}I-9D33 binding and ^{125}I-TSH binding to TSHR coated tubes

Concentration of 90/672[1]	^{125}I-9D33 bound (% total counts added)	Inhibition of ^{125}I-9D33 binding[2] (%)	Inhibition of ^{125}I-TSH binding[2] (%)
40 U/L	4.1	72	92
8 U/L	7.1	52	68
2 U/L	11	28	23
1 U/L	13	10	12
0 U/L	15	0	0

[1]90/672 diluted in a pool of healthy blood donor sera

[2]Inhibition of binding was calculated with the formula used in Table 23

Table 26 Inhibition of ^{125}I-hMAb TSHR1 Fab binding to TSHR coated tubes by polyclonal hMAb TSHR1 anti idiotypic antibodies in sera from a mouse immunised with hMAb TSHR1 Fab

Test sample	Dilution[1] of test sample	% inhibition[2] of ^{125}I-hMAb TSHR1 Fab binding to the TSHR
Assay buffer		0
Sera from a mouse immunised with hMAb TSHR1 Fab	1:100 000 1:50 000 1:10 000 1:5 000 1:1 000	1.3 7.9 49.8 73.0 94.8
Non-immunised mouse serum	1:500	-0.8

[1]Test samples diluted in assay buffer. Binding in the presence of assay buffer was 43%

[2]Inhibition of binding was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{A}{B} \times 100\right]$$

where A = % ^{125}I-hMAb TSHR1 Fab binding in the presence of test sample
B = % ^{125}I-hMAb TSHR1 Fab binding in the presence of assay buffer

Table 27 Inhibition of ^{125}I-hMAb TSHR1 Fab binding to TSHR coated tubes by a mouse monoclonal anti idiotypic antibody 7E51 IgG

Test sample		% binding of ^{125}I-hMAb TSHR1 Fab to the TSHR	% inhibition[1] of ^{125}I-hMAb TSHR1 Fab binding
Assay buffer alone		16.3	0
7E51 IgG diluted in assay buffer	1 µg/mL	14.5	11.0
	10 µg/mL	6.4	60.7
	100 µg/mL	1.7	89.4

[1]see footnote to Table 26